

**EPA REGISTRATION NUMBER 71693-1 – VOLUME 3**



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

05/27/03 02:59 PM

To: Shanaz Bacchus [REDACTED]  
cc: Peter Cotty <pcotty@srcc.ars.usda.gov>, Shanaz  
Bacchus/DC/USEPA/US@EPA  
Subject: RE: AF36 Label

Shanaz

I have included all the changes you requested. The only additional change I made was to spell out the word Worker Protection Standard on the first page.

Let me know if there is anything else that needs attention.

Thanks

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
FAX (732)932-8481  
braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

From: Shanaz Bacchus [REDACTED]  
Sent: Saturday, May 24, 2003 12:13 PM  
To: Mike Braverman  
Cc: Shanaz Bacchus  
Subject: Re: AF36 Label

Attached is a version I inserted the following in Word:

1. 2nd line first page: When applied "to cotton".
2. "Other ingredient:" near wheat seeds in ingredient statement.
3. I did not include this, please do: in "See additional.....statements" below add "on other panel" because Environmental Hazards appear on 2nd page.
- 4a. Over WPS box, please all the language from the page I had given you. If you have misplaced it, look at the Label Review manual for the instructions for that box or email me. Check 40 cfr 156.206(a).
- 4b. I moved the mixer/loader statement into Hazards to humans section. Check 40 CFR 156.212(3)(c). In moving around some of the items, I may have messed up the boxes, so please check them against your last 2-page Cotty version. Do not repeat the statement in the "Directions for Use".
5. Under "Ground Application": 1. Apply Asper.....with a cultivator....." and "DO NOT COVER AF36 COLONIZED WHEAT SEEDS WITH SOIL".
6. Remove the statement "Spray drift....to End-use Product".

If any questions, do email me.

Sorry about all these multiple revisions...just have to deal with all parties' comments.

Have a great Memorial Day weekend. I'll be in the office on Tues, at meetings 10 a.m.-12noon.

Sincerely

\*Personal privacy information\*

Shanaz Bacchus  
703-308-8097

----- Original Message -----

From: "Mike Braverman" <braverman@AESOP.RUTGERS.EDU>  
To: "Shanaz Bacchus" [REDACTED]  
Cc: "Shanaz Bacchus" <Bacchus.Shanaz@epamail.epa.gov>  
Sent: Monday, May 19, 2003 1:43 PM  
Subject: RE: AF36 Label

> Shanaz  
>  
> With help from Peter the label has been reduced to 2 pages without  
> deleting  
> any portion. This will make it easier to fit on the bag.  
>  
> I didn't follow what you were saying about wheat so I didn't change  
> anything related to that. If its still not right, please change in the  
> text  
> so I can see it.  
>  
> I increased the font size of the Caution statement to 18 and the Keep out  
> of  
> reach statement to size 12.  
>  
> The second place that had the word CAUTION is now incorporated into the  
> section of the precautionary statement.  
>  
> Hope this works.  
>  
>  
>  
>  
> Michael Braverman, Ph.D  
> Biopesticide Coordinator  
> IR-4 Project, Rutgers University  
> Technology Centre of New Jersey  
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> braverman@aesop.rutgers.edu  
> IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)  
>  
> -----Original Message-----  
> From: Shanaz Bacchus [REDACTED]  
> Sent: Sunday, May 18, 2003 10:52 AM  
> To: Mike Braverman  
> Subject: Re: AF36 Label  
>  
>  
> Mike, after looking at the label, I observed that the name *Aspergillus*  
> *flavus* is misrepresented when caps are used (the species name "*flavus*"  
> uses  
> lower case. Accordingly, I changed it and italicized the name. Some

\*Personal privacy information\*

minor

- > changes were also made in the text, such as "colonized" wheat seeds (please
- > check if the word "wheat" as included) in order to clarify it is the A.
- > flavus AF36 colonized wheat seeds. Also included goggles, since primary eye
- > irritation study was waived. If later you wish to remove goggles, provide
- > data or information to do so. In the registration notice, you will be asked
- > to change the -R to 1. I will communicate when you have to do this.

Please

- > check the label and let me know if you concur with the changes mentioned
- > above.
- > Thanks

>

>

> ----- Original Message -----

- > From: "Mike Braverman" <braverman@AESOP.RUTGERS.EDU>
- > To: "Shanaz Bacchus" [REDACTED]
- > Sent: Friday, May 16, 2003 3:43 PM
- > Subject: RE: AF36 Label

>

>

- >> Have a nice weekend!

>>

- >> Michael Braverman, Ph.D
- >> Biopesticide Coordinator
- >> IR-4 Project, Rutgers University
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>>

>> -----Original Message-----

- >> From: Shanaz Bacchus [REDACTED]
- >> Sent: Friday, May 16, 2003 3:39 PM
- >> To: Mike Braverman
- >> Subject: Re: AF36 Label

>>

>>

- >> Worked like a charm,
- >> thanks,
- >> shawn

>> ----- Original Message -----

- >> From: "Mike Braverman" <braverman@AESOP.RUTGERS.EDU>
- >> To: <Bacchus.Shanaz@epamail.epa.gov> [REDACTED]
- >> Sent: Friday, May 16, 2003 2:36 PM
- >> Subject: RE: AF36 Label

>>

>>

- >>> Shanaz

>>>

- >>> I hope this attachment works....

\*Personal privacy information\*

>>>

>>>

>>> Michael Braverman, Ph.D

>>> Biopesticide Coordinator

>>> IR-4 Project, Rutgers University

>>> Technology Centre of New Jersey

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>>> -----Original Message-----

>>> From: Bacchus.Shanaz@epamail.epa.gov

>>> [mailto:Bacchus.Shanaz@epamail.epa.gov]

>>> Sent: Friday, May 16, 2003 1:24 PM

>>> To: Mike Braverman

>>> Cc: [REDACTED] Bacchus.Shanaz@epamail.epa.gov; Peter Cotty;

>>> Phil Hutton

>>> Subject: Label

>>>

>>>

>>>

>>> Mike, thanks for the label. I opened in Word.

>>> 1. The reentry statement was missing in the WPS box.

>>> 2. I included a statement near the aerial application to show that no

>>> spray drift is expected, so that no one asks for spray drift

statements.

>>> Please align the statements under the Directions for use and the  
Active

>>> ingredient statement.

>>> 3. In the Furrow irrigation statement: Furrow irrigating.....will

>>> provide....include "will" or the verb.

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>>> Proof and send in 5 final copies, i.e. the ones you would like  
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>>> by FEDEX, as we discussed on the phone this morning. I really wish  
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>>> emailed one would work but our printers don't ever seem to do well  
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>>> your Word documents either at work or home.

>>>

>>> Thanks,

>>>

>>> shawn

>>>

>>>

>



AF362pageCotty may27mb 2003.

\*Personal privacy information\*



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

05/19/03 01:43 PM

To: Shanaz Bacchus [REDACTED]  
cc: Shanaz Bacchus/DC/USEPA/US@EPA  
Subject: RE: AF36 Label

Shanaz

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I didn't follow what you were saying about wheat so I didn't change anything related to that. If its still not right, please change in the text so I can see it.

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The second place that had the word CAUTION is now incorporated into the section of the precautionary statement.

Hope this works.

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Thanks

----- Original Message -----

From: "Mike Braverman" <braverman@AESOP.RUTGERS.EDU>

To: "Shanaz Bacchus" [REDACTED]

Sent: Friday, May 16, 2003 3:43 PM

Subject: RE: AF36 Label

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>

> Michael Braverman, Ph.D

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> From: "Mike Braverman" <braverman@AESOP.RUTGERS.EDU>

> To: <Bacchus.Shanaz@epamail.epa.gov>; [REDACTED]

> Sent: Friday, May 16, 2003 2:36 PM

> Subject: RE: AF36 Label

>

>

>> Shanaz

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>> I hope this attachment works....

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>>

>> Michael Braverman, Ph.D

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>> -----Original Message-----

\*Personal privacy information\*

> > From: Bacchus.Shanaz@epamail.epa.gov  
> > [mailto:Bacchus.Shanaz@epamail.epa.gov]  
> > Sent: Friday, May 16, 2003 1:24 PM  
> > To: Mike Braverman  
> > Cc: [REDACTED] Bacchus.Shanaz@epamail.epa.gov; Peter Cotty;  
> > Phil Hutton  
> > Subject: Label

> >

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> > by FEDEX, as we discussed on the phone this morning. I really wish the

> > emailed one would work but our printers don't ever seem to do well with

> > your Word documents either at work or home.

> >

> > Thanks,

> >

> > shawn

> >

> >



AF362pageCotty may 2003.d



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

05/16/03 02:36 PM

To: Shanaz Bacchus/DC/USEPA/US@EPA, [REDACTED]  
cc:  
Subject: RE: AF36 Label

Shanaz

I hope this attachment works....

Michael Braverman, Ph.D  
Biopesticide Coordinator  
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Sent: Friday, May 16, 2003 1:24 PM  
To: Mike Braverman  
Cc: [REDACTED] Bacchus.Shanaz@epamail.epa.gov; Peter Cotty;  
Phil Hutton  
Subject: Label

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Proof and send in 5 final copies, i.e. the ones you would like stamped, by FEDEX, as we discussed on the phone this morning. I really wish the emailed one would work but our printers don't ever seem to do well with your Word documents either at work or home.

Thanks,

shawn



AF363page label.do

\*Personal privacy information\*



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

05/16/03 12:06 PM

To: [REDACTED] Shanaz Bacchus/DC/USEPA/US@EPA  
cc: Peter Cotty <pcotty@srsc.ars.usda.gov>, Phil  
Hutton/DC/USEPA/US@EPA  
Subject: RE: AF36 analysis/product id and label

Shanaz

I have spoken to Peter Cotty on this issue.

VCG is the only method used and can distinguish AF36 from all other A. flavus strains. It is used both prior to and after production of the batches in a redundant manner.

As stated in MRID 44626101 page 10 ,

" Both isozyme analyses and DNA polymorphisms show that the VCG test specifically identifies the consistently atoxigenic vegetative compatibility group which we call Aspergillus flavus AF36. There is a zero tolerance for A. flavus not identified as Aspergillus flavus AF36."

While isozyme and DNA polymorphisms have been used as a confirmatory technique to the VCG system, there is no intention on using them as part of the QC system. Only VCG will be used.

A complete outline of the identification of the organism is described in MRID 44626101 page 9 in which the AF36 is compared to tester mutants on long term storage which have also been deposited with and available from the American Type Culture Collection.

The updated label is also attached

Michael Braverman, Ph.D  
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-----Original Message-----

From: Bacchus.Shanaz@epamail.epa.gov  
[mailto:Bacchus.Shanaz@epamail.epa.gov]  
Sent: Thursday, May 15, 2003 4:34 PM  
To: Mike Braverman  
Subject: RE: AF36 analysis/product id

As discussed with you on the phone, the only outstanding issue is to find a confirmatory method to identify AF36 apart from VCG analysis.

Please ask Peter if there is any other DNA analysis or some other method which can be used. If necessary, we can teleconference with Peter and John Kough on this issue next week, say Tues.

Thanks,

shawn

703-308-8097



AF363page label.do

**\*Personal privacy information\***



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

05/15/03 02:02 PM

To: Shanaz Bacchus/DC/USEPA/US@EPA, Shanaz Bacchus

cc: Peter Cotty <pcotty@srrc.ars.usda.gov>, Phil  
Hutton/DC/USEPA/US@EPA, Phil Wakelyn <pwakelyn@cotton.org>,  
Larry Anttila <LAnttila@AZcotton.com>

Subject: RE: AF36/label/monitoring

Dear Shanaz

in response to your e-mail we are addressing the following 3 segments.

A) TX efficacy research.

B) Aflatoxin in the batches -HPLC analysis

C) Timeline for storage stability study

A) TX efficacy research.

The FDA does not routinely monitor cottonseed for aflatoxin, it is done at and by the individual gins, oil mills, or cottonseed brokers.

Concerning the efficacy data you are requesting the data will consist of measurements of the displacement of aflatoxin producing strains in soil and on cottonseed by treatments with *Aspergillus flavus* AF36. Air monitoring data will not be included. The incidence of AF36 in the air is not an efficacy question.

As we have stated previously, we do not view this as a public health pesticide and believe that the TX and AZ cotton fields are comparable enough so that performance in Texas is not an issue.

B) Aflatoxin in AF36 batches

In regard to your question about an HPLC method, although HPLC methods exist, TLC and Immunological Tests are routinely used for aflatoxin analyses. These tests are official approved methods for aflatoxin analyses.

As we discussed on several occasions, aflatoxin content of batches is not determined as part of the quality control because it does not provide useful information.

As required by the manufacturing protocol, a batch would be discarded if the vegetative compatibility (VCG) test was negative for AF36. I believe we had

laid this question to rest and that this was already settled, but as an overview here are the quality control procedures:

**1 PRIOR TO PRODUCTION:**

Inoculum of AF36 is transferred from storage and grown in pure culture. Each plate is examined for purity visually on three media. Working Cultures Vials result from these plates. Each culture plate used to make a Working Culture Vial is tested by both vegetative compatibility to ensure the

culture is AF36 and for aflatoxin production. Aflatoxin production is tested by growing the culture in media and under conditions ideal for aflatoxin production. In this process, TLC is used to quantify aflatoxin production. This is a well-established widely accepted technique that has a limit of detection below 1 ppb. This is a redundant test because AF36 has been tested hundreds of times for aflatoxin production and has never been found to produce aflatoxins.

If the VCG test is negative or the TLC test detects aflatoxin the inoculum, the vials would be discarded prior to being accepted as Working Culture Vials.

2. PRODUCTION: During production, the quantity of conidia is increased. Wheat is steamed sterilized, cooled, and the sterile wheat is coated with a suspension of AF36 conidia. The AF36 coated wheat is then incubated for 24 hr. to allow the fungus to briefly colonized the wheat. The wheat is then dried and packaged for use.

### 3. POST PRODUCTION:

Every batch of AF36 colonized wheat produced is tested by VCG analysis to reconfirm the pre-production VCG test.

If these tests were ever negative the batch would be discarded or destroyed.

These procedures prevent the possibility that the product can be contaminated with aflatoxin producers.

All of the above is already submitted in MRID 44626101 page 8

The following is not currently part of the production system, but could be added:

If the POST Production VCG test fails the batch will either be discarded OR analyzed by TLC for aflatoxin.

If the TLC test detects aflatoxin the batch will either be discarded OR analyzed by HPLC.

If these statements would make the protocol acceptable, then these could be added to the production system protocol.

Once again, let me repeat that this is only an operating procedure and in actuality nothing would go past the VCG test of the batch, because if a batch is detected that is negative for AF36 by the vegetative compatibility test, that batch will be discarded.

The aflatoxin content of the sterilized wheat colonized by AF36 (finished product) can be determined as part of the 5 batch analysis. The finished product from each of the batches can be analyzed either in Dr. Cotty's laboratory by TLC or by a commercial laboratory by a different method. However, a requirement of routine aflatoxin analyses, as a component of the manufacturing process would be an unwarranted burden for the grower-run manufacturing facility.

**\*Personal privacy information\***

**C) Timeline for storage stability study**

There is the possibility that product may need to be stored for up to 18 months. As I showed you in the journal article, data has already been published showing 29 months of storage stability, although I understand that you need the stability data on the actual production scale batches. So with an 18-month stability study, time to produce the batches, and time to work with the data and submit a report two and a half years are needed to complete this study.

So in summary, in regard to the additional information needed to reply to and the proposed due dates for the registration:

885.1300 Discussion of Formation of Unintentional Ingredients Human Pathogen, aflatoxin contamination.

Time needed after registration: to be added to the 5-batch analysis. 2 years, 6 months.

885.1400 Analysis of Samples-5 batch analysis including viability and storage stability.

Time needed after registration: 2 yr, 6 months

885.1500 Certification of limits.

Time needed after registration: 2 year

Non-guideline - Efficacy data in Texas

Time needed after registration: 2 years for development of an additional year of data.

Michael Braverman, Ph.D  
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-----Original Message-----

From: Shanaz Bacchus [REDACTED]  
Sent: Monday, May 05, 2003 12:02 PM  
To: Mike Braverman  
Subject: Re: AF36/label/monitoring

Thanks, I'll look at it tomorrow after I get the other stuff done that's desperately needed.

I have another issue to discuss regarding efficacy trials in TX. Can you provide cottonseed data from the FDA monitoring of cottonseed to show that more cottonseed has been acceptable in AZ over the period of the EUP?

**\*Personal privacy information\***

Also, would it be easier for you to provide FDA monitoring of the cottonseed in TX to show that more cottonseed has been acceptable in TX over this season. If so, we may be able to use that data instead of requiring soil and air monitoring data in TX as a condition of registration.

I am just testing the water on this one...and I imagine, so are you. Please discuss with Peter and call me sometime today after 1 p.m., Mon 5/5 on [REDACTED] If not call me on Tues at work (703-308-8097)

Shawn

----- Original Message -----

From: "Mike Braverman" <braverman@AESOP.RUTGERS.EDU>

To: <Bacchus.Shanaz@epamail.epa.gov> [REDACTED]

Cc: "Peter Cotty" <pcotty@srrc.ars.usda.gov>

Sent: Monday, May 05, 2003 11:51 AM

Subject: RE: AF36/label

> Shanaz

>

> I could not make it fit with the other changes/ boxes so I have changed the

> label out of the column format and onto 3 pages. Attached is the latest

> update.

>

> If any other parts of this need attention please let me know.

>

> Thanks

>

> Michael Braverman, Ph.D

> Biopesticide Coordinator

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> -----Original Message-----

> From: Bacchus.Shanaz@epamail.epa.gov

> [mailto:Bacchus.Shanaz@epamail.epa.gov]

> Sent: Saturday, May 03, 2003 11:28 AM

> To: braverman@AESOP.RUTGERS.EDU>

> Subject: AF36/label

>

>

>

> AF36/label

>

> To capture our phone discussion yesterday, Fri 5/2:

>

> 1. Under Caution ( 1st column, 1st para)

> "Harmful if swallowed. Avoid breathing dust. Causes moderate eye

> irritation. Avoid contact with eyes or clothing. Prolonged or

- > frequently....individuals. Wash hands....after handling."
- >
- > 2. User Safety Recommendations (put in box for visibility, indent or align...my email doesn't allow me to do so.)
- >
- > "Users should wash hands before eating, drinking, chewing gum, using tobacco, or using the toilet.
- >
- > Users should remove clothing/PPE (spell out...1st ref) if pesticide gets inside. Then wash thoroughly and put on clean clothing.
- >
- > Users should remove PPE immediately after handling this product. Wash the outside of gloves before removing. As soon as possible, wash thoroughly and change into clean clothing."
- >
- > 3. Above Agric. Use Requirements box: "It is violation...Do not apply..."etc., as per page I gave you for WPS. If you have misplaced it, drop me an email/voicemail, include a fax # and I'll fax it to you...I'm working at home on Monday 5/5, but can access both from home.
- >
- > 4. Ag Use requirements contain statements for early entry workers and REI. Include statements about PPE for mixer/loaders in the Directions for Use.
- >
- > 2nd column
- > 5. Remove 3000 cfu from position near to AF36 active ingredient listing. Put asterisk above AF36 and viability data below Total line to read "> " \*3000 cfu/g End-use Product"
- >
- > 6. Include First Aid Statements in BOx for visibility as per PRN 2001-1 I sent you yesterday p.m. First Aid statements include "If on skin or clothing..."
- >
- > Below First Aid statements:
- >
- > "Have the product container or label with you..."
- >
- > Remove redundancies to tighten up label.
- >
- > I noticed when I converted it from Word to WordPerfect, it became 2 logical pages. The draft label, which we stamp does not have to be the final printed (FP) label. Of course, you don't want to have to do 2 labels, (1 draft and 1 FP) but if it's easier for you to leave the printers the job of rearranging the columns, then the 2 page WP label will be fine, assuming that the content remains the same on the FP.
- >
- > Now that I've kept my promise about the label, have a great weekend, shawn
- > (sent sat 5/3 from home)
- >

\*Personal privacy information\*



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

05/12/03 04:14 PM

To: Shanaz Bacchus [REDACTED]  
cc: Shanaz Bacchus/DC/USEPA/US@EPA, Peter Cotty  
<pcotty@srrc.ars.usda.gov>  
Subject: RE: data waiver requests

Shanaz

I have the waiver requests in the attached file.  
Please let me know if anything else is needed.

Thanks

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
FAX (732)932-8481  
braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

**From:** Shanaz Bacchus [REDACTED]  
**Sent:** Monday, May 12, 2003 2:19 PM  
**To:** Mike Braverman  
**Subject:** data waiver requests

For the (1) acute inhalation study: 152-32: waiver requested because there are no respirable particles in the inerts or End-use Product, inoculated sterilized wheat seeds.

(2) immune response: clearance observed in both acute oral and pulmonary studies ...days, organs, etc.

(3) hypersensitivity study: no hypersensitivity incidents observed in maximally exposed researchers, handlers over experimental phases in lab or field. Also no non-occupational exposure above background levels expected based on agricultural use and for all those other reasons re ubiquitous exposure to naturally occurring organism.

Please put in Data Waiver format and send by email.  
Thanks,  
shawn



301-924-7114 (in case you have any questions, do call) AF36healthwaivers2.wpr



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

05/05/03 11:51 AM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc: Peter Cotty <pcotty@srrc.ars.usda.gov>  
Subject: RE: AF36/label

Shanaz

I could not make it fit with the other changes/ boxes so I have changed the label out of the column format and onto 3 pages. Attached is the latest update.

If any other parts of this need attention please let me know.

Thanks

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
FAX (732)932-8481  
braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

From: Bacchus.Shanaz@epamail.epa.gov  
[mailto:Bacchus.Shanaz@epamail.epa.gov]  
Sent: Saturday, May 03, 2003 11:28 AM  
To: braverman@AESOP.RUTGERS.EDU>  
Subject: AF36/label

AF36/label

To capture our phone discussion yesterday, Fri 5/2:

1. Under Caution (1st column, 1st para)  
"Harmful if swallowed. Avoid breathing dust. Causes moderate eye irritation. Avoid contact with eyes or clothing. Prolonged or frequently....individuals. Wash hands...after handling."

2. User Safety Recommendations (put in box for visibility, indent or align..my email doesn't allow me to do so.)

"Users should wash hands before eating, drinking, chewing gum, using tobacco, or using the toilet.

Users should remove clothing/PPE (spell out..1st ref) if pesticide gets inside. Then wash thoroughly and put on clean clothing.

Users should remove PPE immediately after handling this product. Wash the outside of gloves before removing. As soon as possible, wash

thoroughly and change into clean clothing."

3. Above Agric. Use Requirements box: "It is violation...Do not apply.."etc., as per page I gave you for WPS. If you have misplaced it, drop me an email/voicemail, include a fax # and I'll fax it to you...I'm working at home on Monday 5/5, but can access both from home.

4. Ag Use requirements contain statements for early entry workers and REI. Include statements about PPE for mixer/loaders in the Directions for Use.

2nd column

5. Remove 3000 cfu from position near to AF36 active ingredient listing. Put asterisk above AF36 and viability data below Total line to read " \*3000 cfu/g End-use Product"

6. Include First Aid Statements in BOx for visibility as per PRN 2001-1 I sent you yesterday p.m. First Aid statements include "If on skin or clothing..."

Below First Aid statements:

"Have the product container or label with you..."

Remove redundancies to tighten up label.

I noticed when I converted it from Word to WordPerfect, it became 2 logical pages. The draft label, which we stamp does not have to be the final printed (FP) label. Of course, you don't want to have to do 2 labels, (1 draft and 1 FP) but if it's easier for you to leave the printers the job of rearranging the columns, then the 2 page WP label will be fine, assuming that the content remains the same on the FP.

Now that I've kept my promise about the label, have a great weekend, shawn

(sent sat 5/3 from home)



AF363page label.do



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

05/05/03 08:30 AM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc: [REDACTED]  
Subject: RE: AF36/label

Shanaz

I think under point # 1 it was "Harmful if inhaled", not "Harmful if swallowed" and we put this before the "Avoid breathing dust" statement

So it now looks like this :

Harmful if inhaled avoid breathing dust. Causes moderate eye irritation and avoid contact with eyes, skin or clothing. Prolonged or frequently repeated skin contact may cause allergic reaction in some individuals. Wash hands thoroughly with soap and water after handling.

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
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Tel (732)932-9575 ext 610  
FAX (732)932-8481  
braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

From: Bacchus.Shanaz@epamail.epa.gov  
[mailto:Bacchus.Shanaz@epamail.epa.gov]  
Sent: Saturday, May 03, 2003 11:28 AM  
To: braverman@AESOP.RUTGERS.EDU>  
Subject: AF36/label

AF36/label

To capture our phone discussion yesterday, Fri 5/2:

1. Under Caution (1st column, 1st para)  
"Harmful if swallowed. Avoid breathing dust. Causes moderate eye irritation. Avoid contact with eyes or clothing. Prolonged or frequently....individuals. Wash hands...after handling."
2. User Safety Recommendations (put in box for visibility, indent or align..my email doesn't allow me to do so.)  
  
"Users should wash hands before eating, drinking, chewing gum, using tobacco, or using the toilet.  
  
Users should remove clothing/PPE (spell out..1st ref) if pesticide gets inside. Then wash thoroughly and put on clean clothing.

Users should remove PPE immediately after handling this product. Wash the outside of gloves before removing. As soon as possible, wash thoroughly and change into clean clothing."

3. Above Agric. Use Requirements box: "It is violation...Do not apply.."etc., as per page I gave you for WPS. If you have misplaced it, drop me an email/voicemail, include a fax # and I'll fax it to you...I'm working at home on Monday 5/5, but can access both from home.

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2nd column

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Below First Aid statements:

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Now that I've kept my promise about the label, have a great weekend,  
shawn  
(sent sat 5/3 from home)

\*Personal privacy information\*



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

05/02/03 12:53 PM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc: [REDACTED]  
Subject: RE: Storage stability/AF36

Shanaz

Could you please paste the section of the BRAD pertaining to the additional data requested into an e-mail so that I can construct a letter for Larry Antilla.

Thanks

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
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braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

From: Bacchus.Shanaz@epamail.epa.gov  
[mailto:Bacchus.Shanaz@epamail.epa.gov]  
Sent: Friday, May 02, 2003 11:47 AM  
To: Mike Braverman  
Cc: bacc471@comcast.net  
Subject: Re: Storage stability/AF36

Mike, I just want to make sure I understand the question....are you saying that because the 5th batch will be produced later, that you will need more than 1 year to submit the storage stability data? How much time would be appropriate?

shawn

Phone: 703-308-8097

Mike Braverman  
<braverman@AESOP.R  
UTGERS.EDU> To: Shanaz  
Bacchus/DC/USEPA/US@EPA,  
bacc471@comcast.net  
05/02/03 10:22 AM cc:  
Subject: Storage stability

Shanaz

I was looking over the storage stability portion of what's in the draft BRAD.

What is the storage period needed during such a study? I am thinking about

this in relation to your requirement to do that within a year of a conditional registration. Does the storage stability need to be done on 5

batches? If so the time of production of the 5th batch would be a limiting

factor on when the last study could start.

Please explain.

Thanks

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
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IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

\*Personal privacy information\*



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

05/02/03 12:02 PM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc:  
Subject: RE: Storage stability/AF36

Shanaz

How long does the stability study need to be conducted? 3 month 6 month 1 year?

The 1999 article by Bock and Cotly that I faxed to you on 4/4/03 shows stability over a 29 month period.

Also for the conditional registration itself how long will it be good for?

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
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IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

From: Bacchus,Shanaz@epamail.epa.gov  
[mailto:Bacchus,Shanaz@epamail.epa.gov]  
Sent: Friday, May 02, 2003 11:47 AM  
To: Mike Braverman  
Cc: bacc471@comcast.net  
Subject: Re: Storage stability/AF36

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shawn

Phone: 703-308-8097

Mike Braverman  
<braverman@AESOP.R  
UTGERS.EDU> To: Shanaz  
Bacchus/DC/USEPA/US@EPA,

05/02/03 10:22 AM cc:  
Subject: Storage stability

Shanaz

I was looking over the storage stability portion of whats in the draft BRAD.

What is the storage period needed during such a study? I am thinking about

this in relation to your requirement to do that within a year of a conditional registration . Does the storage stability need to be done on 5

batches? If so the time of production of the 5th batch would be a limiting factor on when the last study could start.

Please explain.

Thanks

Michael Braverman, Ph.D  
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IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

05/02/03 10:22 AM

To: Shanaz Bacchus/DC/USEPA/US@EPA.  
CC:  
Subject: Storage stability

Shanaz

I was looking over the storage stability portion of whats in the draft BRAD. What is the storage period needed during such a study? I am thinking about this in relation to your requirement to do that within a year of a conditional registration . Does the storage stability need to be done on 5 batches? If so the time of production of the 5th batch would be a limiting factor on when the last study could start.

Please explain.

Thanks

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
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IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

04/28/03 10:54 AM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc: Bob Holm <holm@AESOP.RUTGERS.EDU>, Larry Antilla  
<LAntilla@AZcotton.com>, Peter Cotty <pjcotty@srcc.ars.usda.gov>,  
Phil Wakelyn <pwakelyn@cotton.org>, Phil  
Hutton/DC/USEPA/US@EPA, Janet Andersen/DC/USEPA/US@EPA  
Subject: AF36 Final approval process

Dear Shanaz

The cotton folks and IR-4 are really getting excited in anticipation of the  
AF36 approval which we hope will be completed this week.

Thanks for all your efforts in bringing this to a closure. I will be at EPA  
on Wednesday, April 30. I could try to squeeze some time in if there are any  
last minute questions. I have not heard anything since we met on April 15 so  
I assume all is well and on track.

Sincerely

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
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AF362003.ppt



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

04/17/03 03:10 PM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc: Peter Cotty <pcotty@srrc.ars.usda.gov>  
Subject: Updated label

Dear Shanaz

Attached is the updated label. I hope I understood all of the changes requested and we on our way to our approval in May. Its possible that I may be in Crystal City on April 30 on other business, but I'm not sure at this point. Hopefully thats for the signing celebration!

I will be out tomorrow.

Thanks

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
FAX (732)932-8481  
braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)



LABelAF36Sec3NOAfla.doc



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

04/16/03 03:38 PM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc:  
Subject: FW: FW: Aflatoxin Losses

Shanaz

Attached is the benefits statement you requested. Will try to get the label to you tomorrow or Monday.

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
FAX (732)932-8481  
braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

From: Peter Cotty [mailto:pjcotty@srrc.ars.usda.gov]  
Sent: Wednesday, April 16, 2003 3:11 PM  
To: braverman@AESOP.RUTGERS.EDU  
Subject: Re: FW: Aflatoxin Losses

Michael,

It looks good. I made very minor changes to a few sentence. The cost estimate for the ammoniation I reduced some to make it more realistic.

It is good. Thanks for the corrections. I think you should send it on.

--Peter.

>>> Mike Braverman <braverman@AESOP.RUTGERS.EDU> 04/16/03 01:57PM >>>  
Peter

I think you want to remove the reference to Table t-t didn't see it. I changed the order of the whole thing and made a few minor changes. Let me know if the changes I made are OK.

Thanks  
Michael

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390

Tel (732)932-9575 ext 610  
FAX (732)932-8481  
braverman@aesop.rutgers.edu  
IR-4 Website www.cook.rutgers.edu/~ir4

-----Original Message-----

From: Peter Cotty [mailto:pjcotty@srcc.ars.usda.gov]  
Sent: Wednesday, April 16, 2003 10:17 AM  
To: braverman@AESOP.RUTGERS.EDU  
Subject: Aflatoxin Losses

Michael,

Please look this over and let me know if you want it modified.

--Peter.

Peter J. Cotty, Ph.D.  
Research Plant Pathologist  
Southern Regional Research Center  
Agricultural Research Service  
United States Department of Agriculture  
1100 Robert E. Lee Blvd.  
New Orleans, LA 70124

pjcotty@srcc.ars.usda.gov  
Phone: 504-286-4391  
FAX: 504-286-4496

Peter J. Cotty, Ph.D.  
Research Plant Pathologist  
Southern Regional Research Center  
Agricultural Research Service  
United States Department of Agriculture  
1100 Robert E. Lee Blvd.  
New Orleans, LA 70124

pjcotty@srcc.ars.usda.gov  
Phone: 504-286-4391  
FAX: 504-286-4496



Aflatoxin Losses ed.doc



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc: Peter Cotty <pjcotty@srrc.ars.usda.gov>  
Subject: FW: Spore Weights

04/04/03 02:41 PM

Shanaz

Attached I have the information on how the % active ingredient was calculated as in the CSF. I will be faxing some info on storage stability and the 3,000 CFU/gram question.

I hope the FAX on the CFU, Stability, and batches came through. Note: batch info is all the way at the last page.

If I need to put this through front end, let me know.

Thanks

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
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FAX (732)932-8481  
braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

From: Peter Cotty [mailto:pjcotty@srrc.ars.usda.gov]  
Sent: Friday, April 04, 2003 12:01 PM  
To: braverman@AESOP.RUTGERS.EDU  
Subject: Spore Weights

Peter J. Cotty, Ph.D.  
Research Plant Pathologist  
Southern Regional Research Center  
Agricultural Research Service  
United States Department of Agriculture  
1100 Robert E. Lee Blvd.  
New Orleans, LA 70124

pjcotty@srrc.ars.usda.gov  
Phone: 504-286-4391  
FAX: 504-286-4496



Spore Weight in formuation for Label.



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

03/12/03 03:51 PM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc: Bob Holm <holm@AESOP.RUTGERS.EDU>, Peter Cotty  
<pjcotty@srrc.ars.usda.gov>, Phil Hutton/DC/USEPA/US@EPA, Phil  
Wakelyn <pwakelyn@cotlon.org>, Larry Antilla  
<LAntilla@AZcotton.com>  
Subject: RE: Aflatoxin analyses

Shanaz

This is in regard to your inquiry for the relation of AF36 to FDA monitoring data. As I mentioned during our phone conversation and as described in the attached information, AF-36 does not necessarily reduce aflatoxin to a certain level. In this system the proportion of aflatoxin producing fungi are reduced; however, aflatoxin content is not necessarily reduced below FDA action thresholds.

It has been a long journey since the the first submissions by Christina Hartman and Bill Biehn in 1995. We hope this information is useful in explaining the system, negating the need to review the newly requested Texas data. We look forward to discussing how we may come to a timely and successful conclusion on this grower based initiative.

Bob Holm will be joining us for the teleconference tomorrow.

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
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braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

From: Bacchus.Shanaz@epamail.epa.gov  
[mailto:Bacchus.Shanaz@epamail.epa.gov]  
Sent: Monday, March 10, 2003 10:35 AM  
To: Mike Braverman  
Cc: pjcotty@srrc.ars.usda.gov; pwakelyn@cotlon.org;  
LAntilla@AZcotton.com  
Subject: Aflatoxin analyses

Peter, I'm copying you, because I'm aware that Mike's going to be on travel. If you can provide some information to the questions below, it will speed things up. I'm also copying Larry as the registrant, so that he can be aware of the issues which are cropping up.

At issue:

Have any of the studies you submitted actually shown the FDA monitoring data to demonstrate the levels of aflatoxin in cotton are less and within the FDA regulatory levels in treated fields than in untreated? What I envisage with the FDA data is that you will show or have shown

that the levels are within those required by FDA, but that more cotton food commodities were acceptable because treatment during the EUP caused the aflatoxin levels on the food commodity to decrease. Let me explain. The reviewers are arguing that they have not seen the actual aflatoxin level data. They have been told that FDA or whoever is monitoring cotton for market purposes is accepting more of the cotton.

Where are the actual records of the aflatoxin levels that support the decision to accept more for the market? If these data are shown in any of the MRIDs or other submissions, do let me know exactly where to point the reviewers, so that we can save time. If not please send in data to demonstrate that the aflatoxin levels were reduced or are within regulatory levels in cotton after the EUP (i.e. FDA or regulatory monitoring data with xx ppb on cotton seed, cottonseed meal, etc.) Include in the summary what aflatoxin monitoring method was used.

Thanks  
Sincerely,  
Shanaz Bacchus, Chemist  
USEPA/OPP (Mail Code 7511C)  
Biopesticides and Pollution Prevention Division  
1200 Pennsylvania Ave., N.W.  
Washington D.C. 20460  
Phone: 703-308-8097  
Fax: 703-308-7026



AF36Efficacy FDA B raverman version. AF36Pie efficacy slide to MB. LABELAF36Sec3aflatoxin.d



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

03/11/03 12:53 PM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc:  
Subject: FW: FedEx shipment 791549921170

Shanaz

FYI. Hard copy of AF-36 data has been received at EPA

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
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FAX (732)932-8481  
braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

From: sysdeliv@fn3a.prod.fedex.com [mailto:sysdeliv@fn3a.prod.fedex.com]  
Sent: Tuesday, March 11, 2003 12:41 PM  
To: braverman@AESOP.RUTGERS.EDU  
Subject: FedEx shipment 791549921170

Our records indicate that the shipment sent from Michael Braverman/IR-4  
PROJECT/RUTGERS U  
to Shanaz Bacchus/US EPA- BPPD Room 910 has been delivered.  
The package was delivered on 03/11/2003 at 11:51 AM and signed for  
or released by T.BETTES.

The ship date of the shipment was 03/10/2003.

The tracking number of this shipment was 791549921170.

FedEx appreciates your business. For more information about FedEx services,  
please visit our web site at <http://www.fedex.com>

To track the status of this shipment online please use the following:  
[http://www.fedex.com/cgi-bin/tracking?tracknumbers=791549921170&action=track  
&language=english&cntry\\_code=us](http://www.fedex.com/cgi-bin/tracking?tracknumbers=791549921170&action=track&language=english&cntry_code=us)

Disclaimer

-----  
FedEx has not validated the authenticity of any email address.



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

03/07/03 03:00 PM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc:  
Subject: AF-36 efficacy and Aflatoxin data

Shanaz

Thank you for your assistance in moving this forward. AF-36 efficacy and Aflatoxin data are attached. I will FAX the 8570-1 form today and send 3 hard copies on Monday.

Have a good weekend

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
FAX (732)932-8481  
braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)



Efficacy for Texas March 2003b.d

----- Message from "Ramon Jaime" <RJaime@srcc.ars.usda.gov> on Thu, 6 Mar 2003 12:29:29 -0500

-----

To: "Peter Cotty" <pjcotty@srcc.ars.usda.gov>

Subject: aflatoxin paper



aflatoxin in south texas.dc South Texas areas.pd percent over 20.pdf probability of aflatoxin b&w.p



aflatoxin b&w.pdf AF36coverletter2.wpc



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

03/07/03 02:48 PM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc:  
Subject: AF-36 efficacy and Aflatoxin data

Shanaz

Thank you for your assistance in moving this forward. AF-36 efficacy and Aflatoxin data are attached. I will FAX the 8570-1 form today and send 3 hard copies on Monday.

Have a good weekend

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
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braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)



Efficacy for Texas March 2003b.d

----- Message from "Ramon Jaime" <RJaime@srrc.ars.usda.gov> on Thu, 6 Mar 2003 12:29:29 -0500

-----

To: "Peter Cotty" <pjcotty@srrc.ars.usda.gov>

Subject: aflatoxin paper



aflatoxin in south texas.dc South Texas areas.pd percent over 20.pdi probability of aflatoxin b&w.p



aflatoxin b&w.pdf AF36coverletter2.wpc



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

03/04/03 04:15 PM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc:  
Subject: FW: old msg/DW forms

OOPSI here is the attachment

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
FAX (732)932-8481  
braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

From: Mike Braverman [mailto:braverman@aesop.rutgers.edu]  
Sent: Monday, March 03, 2003 2:42 PM  
To: Bacchus.Shanaz@epamail.epa.gov  
Subject: RE: old msg/DW forms

Shanaz

I beleive these were already covered in MRID 4530702, although not in combination with these forms. The data waivers are attached. Do I need to send this through front end as an ammended volume of MRID 45307202 and 45739103?

Please advise.

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
FAX (732)932-8481  
braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

From: Bacchus.Shanaz@epamail.epa.gov  
[mailto:Bacchus.Shanaz@epamail.epa.gov]  
Sent: Thursday, February 27, 2003 7:39 PM  
To: braverman@AESOP.RUTGERS.EDU  
Subject: old msg/DW forms

DW forms again...scroll down to the end of this message.

shawn

----- Forwarded by Shanaz Bacchus/DC/USEPA/US on 02/27/03 02:47 PM -----

Shanaz Bacchus

To: Mike

11/06/02 04:21 PM Braverman@aesop.rutgers.edu

cc:

Subject: Aspergillus eco

data

waiver justification format  
reminder

Mike: Just a reminder, I'm resending the email I had sent 9/20/2002 so that you fill out the forms below for the data waiver request justification for the following ecological effects studies, if you wish to request data waivers for the Section 3 registration:

Wildlife mammalian

Estuarine/marine non targets

Aquatic invertebrates (Daphnia)

Aquatic vertebrate (Fish)

Terrestrial non targets (Plants)

You may refer to previous reviews and submissions submitted for these studies in connection with the EUP as you complete the forms.

Remember to include the reference #s 006456 for the active ingredient and the EPA Reg. # 71693-R in the transmittal letter subject heading.

Adapt the forms to formalize the data waiver requests for health effects. In addition to using the reference #s 006456 for the active ingredient and the EPA Reg. # 71693-R in the transmittal letter subject heading, include the petition # 8E500 t.

If you have any questions, do not hesitate to email/call me.

Sincerely,

shawn

Phone: 703-308-8097

Fax: 703-308-7026

----- Forwarded by Shanaz Bacchus/DC/USEPA/US on 11/06/02 04:04 PM -----

Shanaz Bacchus

To: Mike

09/20/02 12:01 PM Braverman@aesop.rutgers.edu

PM

cc:

Subject: Aspergillus eco data  
waiver justification format

These DW formats are for ecological effects. Please adapt them for health effects....and remember, only health effects for temporary

## Attachment #2: OPPTS Docket Verification and Certification Form



**U. S. ENVIRONMENTAL PROTECTION AGENCY**  
**OFFICE OF PREVENTION, PESTICIDES, AND TOXIC SUBSTANCES**  
**(OPPTS)**

1200 Pennsylvania Avenue, N.W., Washington, D.C. 20460

**DOCKET VERIFICATION AND CERTIFICATION FORM**

For Internal OPPTS Use Only

Title of Action: *Final Rule: Aspergillus flavus AF36; Exemption from the Requirement of a Tolerance*

RIN #: 2070-

Docket ID #: *OPP-2003-0138*

FRL#:

Contact Information:

Name: *Shanaz Bacchus*

Phone: *703-308-8097*

Legacy Information:

*OPP2003-0048, OPP2003-0020*

**Program Lead's Verification:** I have reviewed the docket and verified the following:

- ☐ All of the documents identified in the attached Docket Index have been submitted to the appropriate Docket Manager for inclusion in the docket identified above.
- ☐ Documents containing copyrighted, CBI or otherwise protected information have been identified to allow for "special" processing by the docket.
- ☐ The material has been assembled in a useable form to support the document being published in the FEDERAL REGISTER.

☒ Comments: *no support document*

Date: *4/2/03*

Initials: *Shanaz Bacchus*

Phone: *703-308-8097*

**Docket Manager's Verification and Sign-off:** I hereby confirm the following:

- ☐ The Docket ID # identified above matches our records.
- ☐ The documents identified in the attached Docket Index have been received by the Docket.
- ☐ The documents have been properly processed for inclusion in EPA Dockets, as appropriate.
- ☐ The documents either already are in the docket or are being process for inclusion in the docket.

☒ Comments: *NO Support + DOC.*

Date: *4-2-03*

Signature: *[Signature]*

Phone: *305-6434*

**Program Lead's Certification:** I hereby certify that:

- ☐ I have completed the verification above.
- ☐ I have submitted to the DM all of the documents that I identified needed to be updated, or added to the docket.
- ☐ I have obtained the DM's sign-off.
- ☐ The docket is complete and ready for public release.

☒ Comments: *No support doc*

Date: *4/2/03*

Signature: *Shanaz Bacchus*

Phone: *703-308-8097*

tolerance exemption, but Section 3 registration package needs  
eco/environmental effects DW request.

Hope this helps.

Sincerely,

Shanaz Bacchus, Chemist

USEPA/OPP (Mail Code 7511C)

Biopesticides and Pollution Prevention Division

1200 Pennsylvania Ave., N.W.

Washington D.C. 20460

Phone: 703-308-8097

Fax: 703-308-7026

----- Forwarded by Shanaz Bacchus/DC/USEPA/US on 09/20/02 11:57 AM -----

Zigfridas

Vaituzis

To: Shanaz

Bacchus/DC/USEPA/US@EPA

06/20/02 04:56

cc:

PM

Subject: Aspergillus eco data  
waiver justification format

Shan:

The attached file contains the format that shows how the registrants  
should justify any ecodata waiver requests.

(See attached file: Waivers-format-2.wpd)



AF36Waivers-format-2.wp



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

03/03/03 02:42 PM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc:  
Subject: RE: old msg/DW forms

Shanaz

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Please advise.

Michael Braverman, Ph.D  
Biopesticide Coordinator  
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braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

From: Bacchus.Shanaz@epamail.epa.gov  
[mailto:Bacchus.Shanaz@epamail.epa.gov]  
Sent: Thursday, February 27, 2003 7:39 PM  
To: braverman@AESOP.RUTGERS.EDU  
Subject: old msg/DW forms

DW forms again...scroll down to the end of this message.  
shawn

----- Forwarded by Shanaz Bacchus/DC/USEPA/US on 02/27/03 02:47 PM -----

Shanaz Bacchus

To: Mike  
11/06/02 04:21 PM Braverman@aesop.rutgers.edu  
cc:  
Subject: Aspergillus eco

data

waiver justification format  
reminder

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Aquatic invertebrates (Daphnia)  
Aquatic vertebrate (Fish)  
Terrestrial non targets (Plants)

You may refer to previous reviews and submissions submitted for these studies in connection with the EUP as you complete the forms.  
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Adapt the forms to formalize the data waiver requests for health effects. In addition to using the reference #s 006456 for the active ingredient and the EPA Reg. # 71693-R in the transmittal letter subject heading, include the petition # 8E5001.

If you have any questions, do not hesitate to email/call me.

Sincerely,  
shawn

Phone: 703-308-8097

Fax: 703-308-7026

----- Forwarded by Shanaz Bacchus/DC/USEPA/US on 11/06/02 04:04 PM -----

Shanaz Bacchus

To: Mike

09/20/02 12:01 Braverman@aesop.rutgers.edu

PM

cc:

Subject: Aspergillus eco data  
waiver justification format

These DW formats are for ecological effects. Please adapt them for health effects....and remember, only health effects for temporary tolerance exemption, but Section 3 registration package needs eco/environmental effects DW request.

Hope this helps.

Sincerely,

Shanaz Bacchus, Chemist

USEPA/OPP (Mail Code 7511C)

Biopesticides and Pollution Prevention Division

1200 Pennsylvania Ave., N.W.

Washington D.C. 20460

Phone: 703-308-8097

Fax: 703-308-7026

----- Forwarded by Shanaz Bacchus/DC/USEPA/US on 09/20/02 11:57 AM -----

Zigfridas

Vaituzis

To: Shanaz

Bacchus/DC/USEPA/US@EPA

06/20/02 04:56

cc:

PM

Subject: Aspergillus eco data  
waiver justification format

Shan:

The attached file contains the format that shows how the registrants should justify any ecodata waiver requests.

(See attached file: Waivers-format-2.wpd)



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

03/03/03 08:40 AM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc:  
Subject: RE: reprints

I left them in your cubicle on your chair on Wed 2/26/03 at about 2:30 PM

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
FAX (732)932-8481  
braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

From: Bacchus.Shanaz@epamail.epa.gov  
[mailto:Bacchus.Shanaz@epamail.epa.gov]  
Sent: Saturday, March 01, 2003 10:45 AM  
To: Mike Braverman  
Subject: reprints

Did you bring in the reprints when you were here last Wed/Thurs? Sorry I missed you, I was down with stomach flu and worked at home most of the day on Thurs. If you didn't bring in the reprints, just send them to me by mail...will provide support for the case when I create the final docket.

Thanks

Shanaz Bacchus, Chemist  
USEPA/OPP (Mail Code 7511C)  
Biopesticides and Pollution Prevention Division  
1200 Pennsylvania Ave., N.W.  
Washington D.C. 20460  
Phone: 703-308-8097  
Fax: 703-308-7026



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc:  
Subject: RE: old msg/DW forms

02/28/03 11:17 AM

Shanaz

Got the format. I already have this done but I noticed a few comments on that MRID crosswalk sheet that probably need some further work.

Thanks

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
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braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

From: Bacchus.Shanaz@epamail.epa.gov  
[mailto:Bacchus.Shanaz@epamail.epa.gov]  
Sent: Thursday, February 27, 2003 7:39 PM  
To: braverman@AESOP.RUTGERS.EDU  
Subject: old msg/DW forms

DW forms again...scroll down to the end of this message.  
shawn

----- Forwarded by Shanaz Bacchus/DC/USEPA/US on 02/27/03 02:47 PM -----

Shanaz Bacchus

To: Mike  
11/06/02 04:21 PM Braverman@aesop.rutgers.edu  
cc:  
Subject: Aspergillus eco

data

waiver justification format  
reminder

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Aquatic vertebrate (Fish)  
Terrestrial non targets (Plants)

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If you have any questions, do not hesitate to email/call me.

Sincerely,

shawn

Phone: 703-308-8097

Fax: 703-308-7026

----- Forwarded by Shanaz Bacchus/DC/USEPA/US on 11/06/02 04:04 PM -----

Shanaz Bacchus

To: Mike

09/20/02 12:01 Braverman@aesop.rutgers.edu

PM

cc:

Subject: Aspergillus eco data  
waiver justification format

These DW formats are for ecological effects. Please adapt them for health effects....and remember, only health effects for temporary tolerance exemption, but Section 3 registration package needs eco/environmental effects DW request.

Hope this helps.

Sincerely,

Shanaz Bacchus, Chemist

USEPA/OPP (Mail Code 7511C)

Biopesticides and Pollution Prevention Division

1200 Pennsylvania Ave., N.W.

Washington D.C. 20460

Phone: 703-308-8097

Fax: 703-308-7026

----- Forwarded by Shanaz Bacchus/DC/USEPA/US on 09/20/02 11:57 AM -----

Zigfridas

Vaituzis

To: Shanaz

Bacchus/DC/USEPA/US@EPA

06/20/02 04:56

cc:

PM

Subject: Aspergillus eco data  
waiver justification format

Shan:

The attached file contains the format that shows how the registrants should justify any ecodata waiver requests.

(See attached file: Waivers-format-2.wpd)



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

06/17/03 10:36 AM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc: Peter Cotty <pjcotty@srcc.ars.usda.gov>  
Subject: Af36 cotton forage

Dear Shanaz

In regard to your question about the use of cotton forage and AF36, most cotton is defoliated so cotton forage would be an extremely minor feed item. Cotton leaves and stems would be left in the field and any cleaned out in the ginning process would also be dumped back onto the field. Any other remaining vegetation would be plowed under. Plowing under of all cotton plant debris is mandatory under the boll weevil eradication program. In addition, cotton forage would not influence dietary exposure for the following reasons:

- 1) There would be no secondary transfer of the organism AF36 into milk and milk.
- 2) Mamalian acute oral studies did not indicate any adverse effects and the clearance of AF36 occurred in both rats and birds.
- 3) AF36 is already naturally occurring in soils producing cotton, corn, and wheat so a cow eating any feed that has had any exposure with soil is already consuming AF36.
- 4) AF36 only changes the composition of the *A. flavus* population in soil and subsequently plant debris by decreasing *A. flavus* strains that produce aflatoxin. It does not significantly increase the total *Aspergillus* population
- 5) AF36 is applied as a granule so it is not sprayed onto cotton foliage.

Please let me know if there are any other questions.

Thanks

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
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braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc: Peter Cotty <pcotty@srrc.ars.usda.gov>  
Subject: At EPA 6/18/03

06/17/03 08:56 AM

Shanaz

I will be in meetings with Registration Division in room 315, Crystal Mall 2 on Wed June 18 from 9:30 to 4:30. I will be meeting with Teresa Downs on another submission at 10-11 AM. If we need to meet to discuss anything (or witness the signing?) about AF36 please let me know or come see me. I can leave the meeting if needed. I will only be here until 11:00 today.

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
FAX (732)932-8481  
braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

06/16/03 09:51 AM

To: Peter Cotty <pcotty@src.ars.usda.gov>, Shanaz  
Bacchus/DC/USEPA/US@EPA  
cc: [REDACTED]  
Subject: RE: AF36/cottonseed meal/cottonseed oil

Shanaz

In relation to questions A and B below...

A. See MRID 43763403 page 624 which is from a journal article: Influence of Field Application of an atoxigenic strain of *A. flavus* on the populations of *A. flavus* infecting cotton bolls and on the aflatoxin content of cottonseed.

There is no change in the total population of *A. flavus* compare to the control due to treatment with AF36 only the composition. In addition direct feeding in mamalian acute oral studies showed no adverse affect.

Furthermore, the fungus is killed during oil extraction with organic solvent such as hexane, the leftover part is the cottonseed meal

B. This is what constitutes efficacy. There is no increase in aflatoxin due to treatment with AF36, there is a decrease. See the same article cited above. Aflatoxin is not oil soluble and thats why it stays with the meal.

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
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braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

From: Bacchus.Shanaz@epamail.epa.gov  
[mailto:Bacchus.Shanaz@epamail.epa.gov]  
Sent: Sunday, June 15, 2003 12:16 PM  
To: Mike Braverman  
Subject: RE: AF36/cottonseed meal/cottonseed oil

Please bear with me, because I have not looked at the data submissions and I'm looking for hard data which may be already available.

Does Peter have any data to show that:

a. AF36, the fungus (hyphae, mycelia, conidia, etc.) is not found in cottonseed meal and cottonseed oil? Or if there are data to show that *Aspergillus* is normally found in these food commodities, do they show that the levels of *Aspergillus* found in the controls are not any greater than those treated?

b. aflatoxin levels in these food commodities (cottonseed meal and

cottonseed oil) do not change above background aflatoxin levels as a result of treatment with AF36?

I am preparing the final documents for Janet to send up to Jim Jones for signature. It may be a tight call to give you final word from JJones on Wed. since I plan on putting the whole package on Janet's desk on Tues a.m. However, this week is it. As you can see, I'm working on it today, Sunday, proofing, editing, finalizing. You will get a chance to look at the BRAD before it goes on the Web.

Please call me at home [REDACTED] where I'll be working on Monday morning.

Thanks for your patience and for answering all these nagging questions.

Sincerely,

Shanaz Bacchus, Chemist/RAL

BPPD/OPP



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

06/13/03 10:52 AM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc: [REDACTED]  
Subject: RE: AF36

Shanaz

I pasted Peters previous message below which were the pounds of formulated material. The label states that the formulation contains 0.0008% a.i. so I took the numbers below and multiplied them by 0.000008 to get the pounds of a.i.

Year	Pounds AI
1996	0.00896
1997	0.03704
1998	0.0398
1999	0.8449
2000	1.3601
2001	1.5956
2002	1.5039
2003	1.6
TOTAL	6.99

PETERS PREVIOUS NUMBERS:.....

Following is requested data (as near as I can figure):

Year Amount Used

1996	1,120.00
1997	4,630.00
1998	4,980.00
1999	105,624.00
2000	170,009.00

2001 199,454.00

2002 187,992.00

2003 200,000.00 Projected

1996 to 2003 873,809.00 Total

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
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braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

**From:** Bacchus.Shanaz@epamail.epa.gov [mailto:Bacchus.Shanaz@epamail.epa.gov]

**Sent:** Thursday, June 12, 2003 5:10 PM

**To:** Mike Braverman

**Subject:** Re: AF36

I am sincerely hoping that by June 18 I can hand you a registered label, registration notice, etc. Did you and Peter ever check the # lbs active ingredient used for the experimental years? Is that ~ 0.01 lb ai/acre = 10 lb EP? another way of saying this is: Does the estimate of the lbs EP used during the EUP = 8738.09 lb and does Peter have to manufacture 2000 lb ai for 2003?

So there's a way you can help me now (smile) and please reply ASAP (within the next 24 hrs or less).

Thanks.

Sincerely,

Shanaz Bacchus, Chemist  
USEPA/OPP (Mail Code 7511C)  
Biopesticides and Pollution Prevention Division  
1200 Pennsylvania Ave., N.W.  
Washington D.C. 20460  
Phone: 703-308-8097  
Fax: 703-308-7026

Mike Braverman <braverman@AESOP.RUTGERS.EDU>  
06/12/2003 03:34 PM AST

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc:  
bcc:  
Subject: AF36

Shanaz

I noticed the FR notice on the A. flavus for peanut. How is it looking for AF36. I will be at EPA on June 18th if that helps anything.

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
FAX (732)932-8481  
braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

=



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc:  
Subject: AF36

06/12/03 03:34 PM

Shanaz

I noticed the FR notice on the A. flavus for peanut. How is it looking for AF36. I will be at EPA on June 18th if that helps anything.

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
FAX (732)932-8481  
braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

06/09/03 08:12 AM

To: Shanaz Bacchus/DC/USEPA/US@EPA, Shanaz Bacchus  
[REDACTED]  
cc: Peter Cotty <pcotty@srrc.ars.usda.gov>  
Subject: RE: AF36/lasi laps

Shanaz

These are the numbers I received from Peter

Following is requested data (as near as I can figure):

Year Amount Used

1996 1,120.00

1997 4,630.00

1998 4,980.00

1999 105,624.00

2000 170,009.00

2001 199,454.00

2002 187,992.00

2003 200,000.00 Projected

1996 to 2003 873,809.00 Total

Looking forward to the BRAD

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
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-----Original Message-----

From: Shanaz Bacchus [REDACTED]

Sent: Friday, June 06, 2003 8:11 AM

To: Mike Braverman

**Subject:** AF36/last laps

I hate to be asking questions at the 9th minute, but do you have any estimate of how many pounds of AF36 were used during the EUP?

I'm doing the final edits on the documents and plan to have them on Janet's desk on Tuesday.

From there it's a breeze to Jim Jones, because the team has already concurred. I am now answering lawyer questions...which prompts the question above.

I'm compressed today, but will work on AF36 some of today and on Sunday to get it out as per schedule above.

These are the parts of a package we send up to Janet and Jim Jones:

1. BRAD
2. FR final rule including docket sign-off
3. Fact Sheet
4. Label
5. Your commitment letter

Janet signs the Registration Notice after Jim Jones signs off on 1 and 2 above and we stamp the label, which officially registers the pesticide and enables your tolerance exemption. I will immediately inform you by phone and fax when JJ signs off. I will also send you the BRAD electronically for CBI clearance and putting on the web....so it's got to be perfect.

Does this help? Have a great weekend...

shawn

\*Personal privacy information\*



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

06/02/03 09:11 AM

To: Shanaz Bacchus [REDACTED] Shanaz  
Bacchus/DC/USEPA/US@EPA  
cc: Peter Cotty <pcotty@srrc.ars.usda.gov>  
Subject: RE: NPDES statement/label

Shanaz

A lot of this statement seems redundant, but I beleive all the changes you requested are in the label attached.

Thanks.

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
FAX (732)932-8481  
braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

From: Shanaz Bacchus [REDACTED]  
Sent: Sunday, June 01, 2003 10:08 PM  
To: Mike Braverman; Bacchus.Shanaz@epamail.epa.gov  
Cc: Peter Cotty  
Subject: NPDES statement/label

Mike, this statement ought to be on the label under Environmental Hazards statements. It's for manufacturing use products and called the NPDES statement. In this case, AF36 is both the MUP and the EP. We all missed this...I noticed it as I'm finalizing the BRAD.

I also noticed that there is a redundant spray drift statement: 1 is above the Ag Use directions box and 1 under Environmental Hazard statement. Can you merge these over the Ag Use Box?

"Do not discharge effluent containing this product into lakes, streams, ponds, estuaries, oceans or other waters unless in accordance with the requirements of a National Pollutant Discharge Elimination System (NPDES) permit and the permitting authority has been notified in writing prior to discharge. Do not discharge effluent containing this product to sewer systems without previously notifying the local sewage treatment plant authority. For guidance contact your State Water Board or Regional Office of the EPA."

Looks like you'll have to remove some of the white spaces, and maybe use the Arial narrow font some more.

Shawn

----- Original Message -----

\*Personal privacy information\*

From: "Mike Braverman" <braverman@AESOP.RUTGERS.EDU>  
To: <Bacchus.Shanaz@epamail.epa.gov>  
Cc: "Shanaz Bacchus" [REDACTED] "Peter Cotty"  
<pjcotty@src.ars.usda.gov>  
Sent: Wednesday, May 28, 2003 3:05 PM  
Subject: RE: AF36 Label/arial font

> Shanaz

>

> The label looks fine. Please send me the BRAD for CBI review when available.

>

> Thanks

>

> Michael Braverman, Ph.D

> Biopesticide Coordinator

> IR-4 Project, Rutgers University

> Technology Centre of New Jersey

> 681 U.S. Highway 1 South

> North Brunswick, New Jersey 08902-3390

> Tel (732)932-9575 ext 610

> FAX (732)932-8481

> braverman@aesop.rutgers.edu

> IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

>

> -----Original Message-----

> From: Bacchus.Shanaz@epamail.epa.gov

> [mailto:Bacchus.Shanaz@epamail.epa.gov]

> Sent: Wednesday, May 28, 2003 1:18 PM

> To: Mike Braverman

> Cc: Shanaz Bacchus; Peter Cotty

> Subject: RE: AF36 Label/arial font

>

>

>

> It printed out well. I dropped the R, in order to get it ready for  
> stamping, where we'll just put the 1 in. There was a duplicate "of",  
> which I deleted, in the Ag use box. Also, in that box, I changed the  
> MSHA/NIOSH prefixes to fit in with the order of the abbreviations, i.e.  
> MSHA TC 21 C before the N-95. In the label I added some commas, where  
> appropriate (gives you time to take a breath while reading.....)....hate  
> to be so nit picking...but it's now near perfection (smile)....  
> Now please look at it and let me know that this is the label you want  
> stamped....put my initials near yours.  
> (See attached file: AF362pageCotty may28mbsb 2003narrow.doc)  
> For the other parts of the package:  
> 1. I am proofing the BRAD. As soon as it's signed, I'll send it to you  
> electronically, so that you can read it and certify that there is no CBI  
> in it...then we'll publish it on the internet.  
> 2. The FR office should be sending the typeset final rule soon....and  
> it's getting ready to go up the chain.....  
> It does take a while to pull all pieces together...I had really expected  
> to get it final by now, but some delays were not within BPPD's  
> control...we do deal with different offices apart from OPP...Neither  
> Phil nor I could have made things go any faster.

>  
> Thanks for the quick turnaround.  
> Sincerely,  
> Shanaz Bacchus, Chemist  
> USEPA/OPP (Mail Code 7511C)  
> Biopesticides and Pollution Prevention Division  
> 1200 Pennsylvania Ave., N.W.  
> Washington D.C. 20460  
> Phone: 703-308-8097  
> Fax: 703-308-7026  
>



AF362pageCottyJune2mb2003narrow.



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

05/29/03 09:48 AM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc: Shanaz Bacchus [REDACTED] Peter Cotty  
<pcotty@srcc.ars.usda.gov>  
Subject: RE: ATCC #/Trade mark?

Shanaz

The ATCC# is 96045.

There is no trademark.

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
FAX (732)932-8481  
braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

From: Bacchus.Shanaz@epamail.epa.gov  
[mailto:Bacchus.Shanaz@epamail.epa.gov]  
Sent: Thursday, May 29, 2003 8:44 AM  
To: Mike Braverman  
Cc: Shanaz Bacchus; Peter Cotty  
Subject: ATCC #/Trade mark?

1. More nit picking....what is the ATCC # for AF36?
2. Is AF36 a trade mark? If so, it's not on the label as such...or anywhere else. Do advise.

Thanks.

Shanaz Bacchus, Chemist  
USEPA/OPP (Mail Code 7511C)  
Biopesticides and Pollution Prevention Division  
1200 Pennsylvania Ave., N.W.  
Washington D.C. 20460  
Phone: 703-308-8097  
Fax: 703-308-7026

\*Personal privacy information\*



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

05/28/03 03:05 PM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc: Shanaz Bacchus [REDACTED] Peter Cotty  
<pcotty@src.ars.usda.gov>  
Subject: RE: AF36 Label/arial font

Shanaz

The label looks fine. Please send me the BRAD for CBI review when available.

Thanks

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
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FAX (732)932-8481  
braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

From: Bacchus.Shanaz@epamail.epa.gov  
(mailto:Bacchus.Shanaz@epamail.epa.gov)  
Sent: Wednesday, May 28, 2003 1:18 PM  
To: Mike Braverman  
Cc: Shanaz Bacchus; Peter Cotty  
Subject: RE: AF36 Label/arial font

It printed out well. I dropped the R, in order to get it ready for stamping, where we'll just put the 1 in. There was a duplicate "of", which I deleted, in the Ag use box. Also, in that box, I changed the MSHA/NIOSH prefixes to fit in with the order of the abbreviations, i.e. MSHA TC 21 C before the N-95. In the label I added some commas, where appropriate (gives you time to take a breath while reading.....)....hate to be so nit picking...but it's now near perfection (smile)...

Now please look at it and let me know that this is the label you want stamped....put my initials near yours.

(See attached file: AF362pageCotty may28mbsb 2003narrow.doc)

For the other parts of the package:

1. I am proofing the BRAD. As soon as it's signed, I'll send it to you electronically, so that you can read it and certify that there is no CBI in it...then we'll publish it on the internet.
2. The FR office should be sending the typeset final rule soon....and it's getting ready to go up the chain.....

It does take a while to pull all pieces together...I had really expected to get it final by now, but some delays were not within BPPD's control...we do deal with different offices apart from OPP...Neither Phil nor I could have made things go any faster.

Thanks for the quick turnaround.  
Sincerely,

Shanaz Bacchus, Chemist  
USEPA/OPP (Mail Code 7511C)  
Biopesticides and Pollution Prevention Division  
1200 Pennsylvania Ave., N.W.  
Washington D.C. 20460  
Phone: 703-308-8097  
Fax: 703-308-7026



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

05/28/03 10:24 AM

To: Shanaz Bacchus [REDACTED]  
cc: Peter Cotty <pcotty@srcc.ars.usda.gov>, Shanaz  
Bacchus/DC/USEPA/US@EPA  
Subject: RE: AF36 Label/arial font

Shanaz

Here it is with a narrow font. From what I remember you had trouble viewing or printing this at work. The content is not changed, just the font. I have not changed the font on the critical size words such as caution or hazard statement.

Hope this helps with the margins

Thanks

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
FAX (732)932-8481  
braverman@aesop.rutgers.edu  
IR-4 Website www.cook.rutgers.edu/~ir4

-----Original Message-----

From: Shanaz Bacchus [REDACTED]  
Sent: Tuesday, May 27, 2003 4:40 PM  
To: Mike Braverman  
Cc: Peter Cotty; Shanaz Bacchus  
Subject: Re: AF36 Label/arial font

I forgot to add, did you try using arial narrow font for everything to fit and give you some margins?

shawn

----- Original Message -----

From: "Mike Braverman" <braverman@AESOP.RUTGERS.EDU>  
To: "Shanaz Bacchus" [REDACTED]  
Cc: "Peter Cotty" <pcotty@srcc.ars.usda.gov>; "Shanaz Bacchus" <Bacchus.Shanaz@epamail.epa.gov>  
Sent: Tuesday, May 27, 2003 2:59 PM  
Subject: RE: AF36 Label

> Shanaz

>

> I have included all the changes you requested. The only additional change

I

> made was to spell out the word-Worker Protection Standard on the first page.

>

> Let me know if there is anything else that needs attention.

\*Personal privacy information\*

>  
> Thanks  
>  
> Michael Braverman, Ph.D  
> Biopesticide Coordinator  
> IR-4 Project, Rutgers University  
> Technology Centre of New Jersey  
> 681 U.S. Highway 1 South  
> North Brunswick, New Jersey 08902-3390  
> Tel (732)932-9575 ext 610  
> FAX (732)932-8481  
> braverman@aesop.rutgers.edu  
> IR-4 Website www.cook.rutgers.edu/~ir4  
>  
> -----Original Message-----  
> From: Shanaz Bacchus [REDACTED]  
> Sent: Saturday, May 24, 2003 12:13 PM  
> To: Mike Braverman  
> Cc: Shanaz Bacchus  
> Subject: Re: AF36 Label  
>  
>  
> Attached is a version I inserted the following in Word:  
> 1. 2nd line first page: When applied "to cotton".  
> 2. "Other ingredient:" near wheat seeds in ingredient statement.  
> 3. I did not include this, please do: in "See additional.....statements"  
> below add "on other panel" because Environmental Hazards appear on 2nd  
> page.  
> 4a. Over WPS box, please all the language from the page I had given you.  
> If you have misplaced it, look at the Label Review manual for the  
> instructions for that box or email me. Check 40 cfr 156.206(a).  
> 4b. I moved the mixer/loader statement into Hazards to humans section.  
> Check 40 CFR 156.212(3)(c). In moving around some of the items, I may  
> have  
> messed up the boxes, so please check them against your last 2-page Colty  
> version. Do not repeat the statement in the "Directions for Use".  
> 5. Under "Ground Application": 1. Apply Asper.....with a  
> cultivator....." and "DO NOT COVER AF36 COLONIZED WHEAT SEEDS WITH SOIL".  
> 6. Remove the statement "Spray drift....to End-use Product".  
>  
> If any questions, do email me.  
> Sorry about all these multiple revisions...just have to deal with all  
> parties' comments.  
> Have a great Memorial Day weekend. I'll be in the office on Tues, at  
> meetings 10 a.m.-12noon.  
> Sincerely  
> Shanaz Bacchus  
> 703-308-8097  
>  
> ----- Original Message -----  
> From: "Mike Braverman" <braverman@AESOP.RUTGERS.EDU>  
> To: "Shanaz Bacchus" [REDACTED]  
> Cc: "Shanaz Bacchus" <Bacchus.Shanaz@epamail.epa.gov>  
> Sent: Monday, May 19, 2003 1:43 PM  
> Subject: RE: AF36 Label  
>

\*Personal privacy information\*

>  
> > Shanaz  
> >  
> > With help from Peter the label has been reduced to 2 pages without  
> deleting  
> > any portion. This will make it easier to fit on the bag.  
> >  
> > I didn't follow what you were saying about wheat so I didn't change  
> > anything related to that. If its still not right, please change in the  
> text  
> > so I can see it.  
> >  
> > I increased the font size of the Caution statement to 18 and the Keep  
out  
> of  
> > reach statement to size 12.  
> >  
> > The second place that had the word CAUTION is now incorporated into the  
> > section of the precautionary statement.  
> >  
> > Hope this works.  
> >  
> >  
> >  
> >  
> > Michael Braverman, Ph.D  
> > Biopesticide Coordinator  
> > IR-4 Project, Rutgers University  
> > Technology Centre of New Jersey  
> > 681 U.S. Highway 1 South  
> > North Brunswick, New Jersey 08902-3390  
> > Tel (732)932-9575 ext 610  
> > FAX (732)932-8481  
> > braverman@aesop.rutgers.edu  
> > IR-4 Website www.cook.rutgers.edu/~ir4  
> >  
> > -----Original Message-----  
> > From: Shanaz Bacchus  
> > Sent: Sunday, May 18, 2003 10:52 AM  
> > To: Mike Braverman  
> > Subject: Re: AF36 Label  
> >  
> >  
> > Mike, after looking at the label, I observed that the name Aspergillus  
> > flavus is misrepresented when caps are used (the species name "flavus"  
> uses  
> > tower case. Accordingly, I changed it and italicized the name. Some  
> minor  
> > changes were also made in the text, such as "colonized" wheat seeds  
> (please  
> > check if the word "wheat" as included) in order to clarify it is the A.  
> > flavus AF36 colonized wheat seeds. Also included goggles, since primary  
> eye  
> > irritation study was waived. If later you wish to remove goggles,  
provide  
> > data or information to do so. In the registration notice, you will be

\*Personal privacy information\*

> asked  
>> to change the -R to 1. I will communicate when you have to do this.  
> Please  
>> check the label and let me know if you concur with the changes mentioned  
>> above.  
>> Thanks  
>>  
>>  
>> ----- Original Message -----  
>> From: "Mike Braverman" <braverman@AESOP.RUTGERS.EDU>  
>> To: "Shanaz Bacchus" [REDACTED]  
>> Sent: Friday, May 16, 2003 3:43 PM  
>> Subject: RE: AF36 Label  
>>  
>>  
>>> Have a nice weekend!  
>>>  
>>> Michael Braverman, Ph.D  
>>> Biopesticide Coordinator  
>>> IR-4 Project, Rutgers University  
>>> Technology Centre of New Jersey  
>>> 681 U.S. Highway 1 South  
>>> North Brunswick, New Jersey 08902-3390  
>>> Tel (732)932-9575 ext 610  
>>> FAX (732)932-8481  
>>> braverman@aesop.rutgers.edu  
>>> IR-4 Website www.cook.rutgers.edu/~ir4  
>>>  
>>> -----Original Message-----  
>>> From: Shanaz Bacchus [REDACTED]  
>>> Sent: Friday, May 16, 2003 3:39 PM  
>>> To: Mike Braverman  
>>> Subject: Re: AF36 Label  
>>>  
>>>  
>>> Worked like a charm,  
>>> thanks,  
>>> shawn  
>>> ----- Original Message -----  
>>> From: "Mike Braverman" <braverman@AESOP.RUTGERS.EDU>  
>>> To: <Bacchus.Shanaz@epamail.epa.gov>; [REDACTED]  
>>> Sent: Friday, May 16, 2003 2:36 PM  
>>> Subject: RE: AF36 Label  
>>>  
>>>  
>>>> Shanaz  
>>>>  
>>>> I hope this attachment works....  
>>>>  
>>>>  
>>>> Michael Braverman, Ph.D  
>>>> Biopesticide Coordinator  
>>>> IR-4 Project, Rutgers University  
>>>> Technology Centre of New Jersey  
>>>> 681 U.S. Highway 1 South  
>>>> North Brunswick, New Jersey 08902-3390

\*Personal privacy information\*

>>>> Tel (732)932-9575 ext 610  
>>>> FAX (732)932-8481  
>>>> braverman@aesop.rutgers.edu  
>>>> IR-4 Website www.cook.rutgers.edu/~ir4  
>>>>  
>>>> -----Original Message-----  
>>>> From: Bacchus.Shanaz@epamail.epa.gov  
>>>> [mailto:Bacchus.Shanaz@epamail.epa.gov]  
>>>> Sent: Friday, May 16, 2003 1:24 PM  
>>>> To: Mike Braverman  
>>>> Cc: [REDACTED] Bacchus.Shanaz@epamail.epa.gov; Peter  
Cotty;  
>>>> Phil Hutton  
>>>> Subject: Label  
>>>>  
>>>>  
>>>>  
>>>> Mike, thanks for the label. I opened in Word.  
>>>> 1. The reentry statement was missing in the WPS box.  
>>>> 2. I included a statement near the aerial application to show that  
no  
>>>> spray drift is expected, so that no one asks for spray drift  
> statements.  
>>>> Please align the statements under the Directions for use and the  
> Active  
>>>> ingredient statement.  
>>>> 3. In the Furrow irrigation statement: Furrow irrigating.....will  
>>>> provide.....include "will" or the verb.  
>>>>  
>>>> Proof and send in 5 final copies, i.e. the ones you would like  
> stamped,  
>>>> by FEDEX, as we discussed on the phone this morning. I really wish  
> the  
>>>> emailed one would work but our printers don't ever seem to do well  
> with  
>>>> your Word documents either at work or home.  
>>>>  
>>>> Thanks,  
>>>>  
>>>> shawn  
>>>>  
>>>>  
>>  
>



AF362pageCotty may28mb 2003narrow.



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

05/28/03 08:03 AM

To: Shanaz Bacchus [REDACTED]  
cc: Peter Cotty <pcotty@src.ars.usda.gov>, Shanaz  
Bacchus/DC/USEPA/US@EPA  
Subject: RE: AF36 commitment letter

Shanaz

I understood that Larry had faxed it on Friday, and also sent it Fedex but I have asked him to refax just to be sure.

FYI the outline draft of the letter that I asked Larry to put on his letterhead is attached. I assume the letter he sent was identical or very similar.

Please let us know if you receive his fax.

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
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braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

From: Shanaz Bacchus [REDACTED]  
Sent: Tuesday, May 27, 2003 8:47 PM  
To: Mike Braverman  
Cc: Peter Cotty; Shanaz Bacchus  
Subject: Re: AF36 commitment letter

Have you sent it in yet? I need to provide the commitment letter with the package for Office Director signature. If not, please have Larry fax me a copy ASAP. If you have already sent it, then do ignore this message.

Thanks,  
shawn



AF36conditionalDennis Szuhay.r

aggregate exposure to *Aspergillus flavus* AF36.

2. Infants and children. Based on the lack of toxicity and natural occurrence, there is reasonable certainty that no harm to infants, children, or adults will result from aggregate exposure to *Aspergillus flavus* AF36. Exempting *Aspergillus flavus* AF36 from the requirement of a tolerance should pose no significant risk to humans or the environment.

#### G. Effects on the Immune and Endocrine Systems

*Aspergillus flavus* AF36 is a naturally occurring organism, which does not produce aflatoxin, and is thus safer than the *Aspergillus flavus* isolates that produce aflatoxin. To date there is no evidence to suggest that *Aspergillus flavus* AF36 functions in a manner similar to any known hormone, or that it acts as an endocrine disrupter.

#### H. Efficacy

Existence of aflatoxins in the environment is a public health hazard. Data were submitted to demonstrate that proper use of *Aspergillus flavus* AF36 results in reductions in the average aflatoxin producing potential of fungi resident in treated areas and in reductions in the quantity of aflatoxins in crops. In field tests prior to 1996, the aflatoxin content of cottonseed was shown to be inversely related to the proportion of the *Aspergillus flavus* community on the crop composed of *Aspergillus flavus* AF36. Detailed analyses of the aflatoxin content of commercial fields from 1996 through 1998 confirmed that reduced aflatoxin levels were associated with displacement of aflatoxin producers by *Aspergillus flavus* AF36 from treated crops and that treatments were associated with up to 90% reductions in crop aflatoxin content.

Efficacy of applications of *Aspergillus flavus* AF36 in displacing aflatoxin producers was demonstrated for fungal communities both on cottonseed from treated crops at harvest and in soils of treated fields 1 year after treatment. This included cotton crops treated in 1996 (112 acres treated), 1997 (463 acres treated), 1998 (499 acres), 1999 (10,488 acres), 2000 (16,725 acres), and 2001 (19,975 acres treated). The proportion of *Aspergillus flavus* communities composed of *Aspergillus flavus* AF36 indicates the extent to which aflatoxin producers were displaced. In 1996 average incidence of AF36 on treated crops was 88.5% and in the soil, 1 year after treatment, incidence of AF36 was 85.2%. Incidences of AF36 on treated crops were 78% and 67% in 1997 and 1998, respectively, and in soil 1 year after treatment, AF36 incidences were 72% and 77%, respectively. Successful displacement was also observed as the acreage treated rapidly expanded from 1999 to 2001 with average incidences of AF36 on treated crops ranging from 57% in 1999 to 66% in 2001.

Aflatoxin-producing S strain isolates of *Aspergillus flavus* are prominent in soils of cotton producing areas of Arizona and south Texas. They produce more aflatoxins than other *Aspergillus flavus* isolates such as the non-aflatoxin-producing L strain *Aspergillus flavus* AF36. Applications of AF36 during the experimental program were effective at displacing the high aflatoxin producing S strain of *Aspergillus flavus*. During the course of the experimental use program, *Aspergillus flavus* AF36 also caused long-term reductions in the aflatoxin producing potential of fungal communities in agricultural

fields. *Aspergillus flavus* AF36 retained atoxigenicity (failure to produce aflatoxins) upon repeated reisolation from treated fields 1, 2, or 3 years after treatment. Thus, there was a long-term reduction in the potential of fungal communities to produce aflatoxins in treated areas. The average aflatoxin producing potential of *Aspergillus flavus* communities resident in soils of treated fields was reduced on average 73% 1 year after treatment over the 3 year period (1996 to 1999). S strain isolates, which produced very high levels of aflatoxins, with field averages ranging from 7,100 ppb, aflatoxin to 22,700 ppb, aflatoxin, were effectively displaced. Their incidence was reduced from initially composing 46% of *Aspergillus flavus* soil communities to composing on average of 11%.

#### I. Existing Tolerances

The registrant is not aware of any existing tolerances or tolerance exemptions for *Aspergillus flavus* AF36, other than the temporary tolerance exemption on cotton (40 CFR 180.1206) in conjunction with an EUP, which expires on December 30, 2004.

#### J. International Tolerances

There are no Codex maximum residue levels established for residues of *Aspergillus flavus* AF36. *Aspergillus flavus* AF36 containing products are presently not registered for pest control outside of the United States.

(FR Doc. 03-3696 Filed 2-13-03; 8:45 am]  
BILLING CODE 6560-50-S

-----  
You are currently subscribed to epa-pest as: BRAVERMAN@AESOP.RUTGERS.EDU

To unsubscribe, send a blank email to leave-epa-pest-48172G@lists.epa.gov

OR:

Use the listserver's web interface at <https://lists.epa.gov/cgi-bin/lyris.pl> to manage your subscription.

For problems with this list, contact epa-pest-Owner@lists.epa.gov  
-----



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc:  
Subject: RE:Cotton/Lygus

02/13/03 02:11 PM

Shanaz

By "interest" do you mean involvement in developing the technology, or do you mean support of the concept.

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
FAX (732)932-8481  
braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

From: Bacchus.Shanaz@epamail.epa.gov  
[mailto:Bacchus.Shanaz@epamail.epa.gov]  
Sent: Thursday, February 13, 2003 11:56 AM  
To: Mike Braverman  
Cc: Jamerson.Hoyt@epamail.epa.gov; kunkel@AESOP.RUTGERS.EDU;  
Forrest.Robert@epamail.epa.gov; Jackson.Sidney@epamail.epa.gov  
Subject: RE: Follow Up Issues to Alfalfa Growers Group Meeting/pest

Mike, do you know if there is any ARS interest in a transgenic cotton for control of lygus bugs?

Shanaz Bacchus, Chemist  
USEPA/OPP (Mail Code 7511C)  
Biopesticides and Pollution Prevention Division  
1200 Pennsylvania Ave., N.W.  
Washington D.C. 20460  
Phone: 703-308-8097  
Fax: 703-308-7026

Mike Braverman  
<braverman@AESOP.RUTGERS.EDU>  
02/12/03 02:00 PM  
To: Sidney  
Jackson/DC/USEPA/US@EPA,  
kunkel@AESOP.RUTGERS.EDU  
cc: Shanaz  
Bacchus/DC/USEPA/US@EPA,

Hoyt

Robert

Jamerson/DC/USEPA/US@EPA,  
Forrest/DC/USEPA/US@EPA

Subject: RE: Follow Up  
Alfalfa Growers Group

Sidney

Sorry I sent a blank before. My computer got ahead of me.

Since there was interest in Prohexadione-calcium which is a plant growth regulator, I assume that is the type of product that they are interested in

One of the products Dan sent to you from our list *Bacillus cereus* is registered as a PGR on cotton.  
[http://www.epa.gov/pesticides/biopesticides/ingredients/factsheets/factsheet\\_119801.htm](http://www.epa.gov/pesticides/biopesticides/ingredients/factsheets/factsheet_119801.htm)

I did not find *Bacillus cereus* as an individual product , but it is a component of Pix Plus which is labeled on cotton. The Mepiquat chloride component is not a biopesticide

<http://www.cdms.net/manuf/1prod.asp?pd=3706&lc=0>

We do not have anything specific for alfalfa, although many biopesticides are labeled broadly and may contain alfalfa on the label. If there is a specific pest that they are interested in please let me know.

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
FAX (732)932-8481  
braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

From: Jackson.Sidney@epamail.epa.gov  
[mailto:Jackson.Sidney@epamail.epa.gov]  
Sent: Tuesday, February 11, 2003 1:22 PM  
To: kunket@AESOP.RUTGERS.EDU; braverman@AESOP.RUTGERS.EDU  
Cc: Bacchus.Shanaz@epamail.epa.gov; Jamerson.Hoyt@epamail.epa.gov;  
Forrest.Robert@epamail.epa.gov  
Subject: Follow Up Issues to Alfalfa Growers Group Meeting

Dan/Mike:

I had an opportunity to participate in a meeting today with reps. from subject Group. I spoke briefly on relevant current alfalfa pesticide/regulatory matters including zinc phosphide and prohexadione calcium pesticide regulatory schedules/initiatives, and mixed stands: alfalfa-grasses tolerance considerations, CropLife/IR-4 interests and efforts, etc. I need your help regarding the following issues that came up and hope that you will respond/ follow up with listed contacts and/or advise me.

1. Dan, would you have the appropriate Director follow up? What pests are targeted by the seed treatment pesticide, prohexadione calcium? I was unable to locate that information on IR-4's website which shows the chemical slated for IR-4 research project - residue study.

This inquiry came from Mr. Mark Wagoner of Wagoner Touchet Farms, Inc., Touchet, WA. Phone # is 509-394-2970, cell: 509-520-1230, e-mail: wagoner@pocketinet.com.

2. Mike - Are there specific biopesticide research initiatives/controls IR-4 has planned or under development as primary or IPM pest controls for use on alfalfa (seed or forage)? What, if any, are potential regulatory issues?

Inquiry from Ms. Sharie Fitzpatrick, Director of Regulatory Affairs & QA for Biotech Traits, Forage Genetics Int'l, West Salem, WI, phone 608-786-2121, e-mail: sfitzpatrick@foragegenetics.com

Also, Mr. Ross Nishihara wanted to know if IR-4's annual budget has been approved and what it is? This info may be found on your web page which I referred participants to for specific IR-4 information. Mr. Ross is with Ridgeview Farms, Inc., Adrian, OR, phone 541-339-4931, e-mail: rnish@widaho.net.

Thanks for your help. Sidney

Printed on Recycled Paper

EPA Form 1020-1A (1/90)

OFFICIAL FILE COPY

DATE							
SURNAME							
SYMBOL							

Mike Braverman  
braverman@AESOP.RUTGERS.EDU>  
02/12/03 02:00 PM

To: Sidney Jackson/DC/USEPA/US@EPA,  
Kunkel@AESOP.RUTGERS.EDU  
cc: Shanaz Bacchus/DC/USEPA/US@EPA, Hoyt  
Jamerson/DC/USEPA/US@EPA, Robert  
Forrest/DC/USEPA/US@EPA  
Subject: RE: Follow Up Issues to Alfalfa Growers Group Meeting

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Michael Braverman, Ph.D  
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IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
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Tel (732)932-9575 ext 610  
FAX (732)932-8481  
braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

From: Jackson.Sidney@epamail.epa.gov  
[mailto:Jackson.Sidney@epamail.epa.gov]  
Sent: Tuesday, February 11, 2003 1:22 PM  
To: kunkel@AESOP.RUTGERS.EDU; braverman@AESOP.RUTGERS.EDU  
Cc: Bacchus.Shanaz@epamail.epa.gov; Jamerson.Hoyt@epamail.epa.gov;  
Forrest.Robert@epamail.epa.gov  
Subject: Follow Up Issues to Alfalfa Growers Group Meeting

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Thanks for your help. Sidney

DATE							
SURNAME							
SYMBOL							

Mike Braverman  
mbraverman@AESOP.  
RUTGERS.EDU>

02/12/03 01:20 PM

To: Dan Kunkel [mailto:kunkel@AESOP.RUTGERS.EDU], Sidney  
Jackson [mailto:sidney@epa.gov]

cc: Shanaz Bacchus/DC/USEPA/US@EPA, Hoyt  
Jamerson/DC/USEPA/US@EPA, Robert  
Forrest/DC/USEPA/US@EPA, wagoner@pocketinet.com,  
stfzpatrick@foragegenetics.com, rnish@widaho.net

Subject: RE: Follow Up Issues to Alfalfa Growers Group Meeting

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
FAX (732)932-8481  
braverman@aesop.rutgers.edu  
IR-4 Website www.cook.rutgers.edu/~ir4

-----Original Message-----

From: Dan Kunkel [mailto:kunkel@AESOP.RUTGERS.EDU]  
Sent: Tuesday, February 11, 2003 5:14 PM  
To: Jackson.Sidney@epamail.epa.gov; braverman@AESOP.RUTGERS.EDU  
Cc: Bacchus.Shanaz@epamail.epa.gov; Jamerson.Hoyt@epamail.epa.gov,  
Forrest.Robert@epamail.epa.gov; wagoner@pocketinet.com;  
stfzpatrick@foragegenetics.com; rnish@widaho.net  
Subject: RE: Follow Up Issues to Alfalfa Growers Group Meeting

Hello Sidney,  
Thanks for the information.  
Prohexadione calcium is a Plant Growth regulator that BASF has. It has been  
used in fruit trees to decrease the amount of pruning. As well it helps to  
remove some diseases, as air flow through the trees is better. Although we  
have not started any research with this product, we have received request.  
From our database it looks like the use for alfalfa would be to increase  
leaves and probably make the alfalfa a better feed for livestock.

Regarding the biopesticides, I am attaching a printout of all of our  
biopesticide projects with alfalfa. Michael is out of the office this week  
and will likely respond with more information on his return.

Regarding our budget, no it has not been approved. We could get an  
increase, decrease or stay the same..... - last year we received ca. \$10.5  
Million from our CSREES grant.

Best regards,  
Dan

Daniel L. Kunkel, Ph D.  
IR-4 Project  
Rutgers University  
732/932-9575x616  
<http://pestdata.ncsu.edu/ir-4/>

-----Original Message-----

From: Jackson.Sidney@epamail.epa.gov  
[mailto:Jackson.Sidney@epamail.epa.gov]  
Sent: Tuesday, February 11, 2003 1:22 PM  
To: kunkel@AESOP.RUTGERS.EDU; braverman@AESOP.RUTGERS.EDU  
Cc: Bacchus.Shanaz@epamail.epa.gov; Jamerson.Hoyt@epamail.epa.gov;  
Forrest.Robert@epamail.epa.gov  
Subject: Follow Up Issues to Alfalfa Growers Group Meeting

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Inquiry from Ms. Sharie Fitzpatrick, Director of Regulatory Affairs & QA for Biotech Traits, Forage Genetics Int'l, West Salem, WI, phone 608-786-2121, e-mail: sfitzpatrick@foragegenetics.com

Also, Mr. Ross Nishihara wanted to know if IR-4's annual budget has been approved and what it is? This info may be found on your web page which I referred participants to for specific IR-4 information. Mr. Ross is with Ridgeview Farms, Inc., Adrian, OR, phone 541-339-4931, e-mail: rnish@widaho.net.

Thanks for your help. Sidney

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

CONCURRENCES

SYMBOL								
SURNAME								
DATE								

\*Personal privacy information\*



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

02/06/03 09:14 AM

To: Shanaz Bacchus/DC/USEPA/US@EPA, Peter Cotty  
<picotty@src.ars.usda.gov>

cc: Phil Wakelyn <pwakelyn@colton.org>, Larry Antilla  
<LAntilla@AZcolton.com>

Subject: FW: NOF's moving along

Shanaz

Thanks.

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
FAX (732)932-8481  
braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

From: Shanaz Bacchus [REDACTED]  
Sent: Thursday, February 06, 2003 8:49 AM  
To: Mike Braverman  
Subject: NOF's moving along

Great news!

Phil just called that he's signed off on the FR Notice of Filing and sent it up for fund appropriation.

Have a great day!  
Shanaz Bacchus



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc: Peter Cotty <pcotty@srrc.ars.usda.gov>  
Subject: RE: Final Rule

02/05/03 04:10 PM

Shanaz

I'm not sure what you are asking for. I faxed a copy of the docket index/release letter to you on Dec 20. Attached is the cover letter dated Dec 23, 2002 to which I attached the signed original. Do you now need an electronic version of the same information?

Does it need to be repeated for the final rule versus the notice?

When is the notice to be coming out in the FR ?

You also mentioned something last week about the Authorization letters. Those are below in Word Format. Please tell me how to modify if needed

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
FAX (732)932-8481  
braverman@aesop.rutgers.edu  
IR-4 Website www.cook.rutgers.edu/~ir4

-----Original Message-----

From: Bacchus.Shanaz@epamail.epa.gov  
[mailto:Bacchus.Shanaz@epamail.epa.gov]  
Sent: Wednesday, February 05, 2003 3:13 PM  
To: Mike Braverman  
Subject: Final Rule

To get ready for the final rule, assuming that all goes well with the rest of the reviews, please get the docket release letter ready. This would involve classifying the efficacy and toxicology studies as non-CBI. If you have any questions about which ones should be releasable to the public, please call John Kough.

Can you use the letter I had sent you for the release for the notice of filing, and insert the answers electronically so that we can put the list of studies as an electronic file on the net and not have to scan letters with handwritten notes? Also, please send me a hard copy with original signatures. Check the number of pages to make sure that the pagination is correct. (Rals, like registrants, now have to paginate things for the docket!)

Thanks

Shanaz Bacchus, Chemist  
USEPA/OPP (Mail Code 7511C)  
Biopesticides and Pollution Prevention Division  
1200 Pennsylvania Ave., N.W.  
Washington D.C. 20460  
Phone: 703-308-8097  
Fax: 703-308-7026



AF36coverletterDocIndex.wf AF36sec3AntillaAndersen.doc AF36sec3cottyAndersen.doc



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

01/14/03 11:05 AM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc: Peter Cotty <picotty@src.ars.usda.gov>  
Subject: FW: NOF AF36 includes Efficacy for docket prep/please comment

Shanaz

As per your request, I have discussed the aflatoxin reduction issue with Peter. Please see section H, bottom of page 11 in red color which contains 2 new sentences.

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
FAX (732)932-8481  
braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

From: Mike Braverman [mailto:braverman@aesop.rutgers.edu]  
Sent: Friday, January 10, 2003 4:05 PM  
To: Bacchus.Shanaz@epamail.epa.gov  
Cc: Peter Cotty; Hutton.Phil@epamail.epa.gov;  
Kough.John@epamail.epa.gov; etsitty.carl@epamail.epa.gov;  
Tomimatsu.Gail@epamail.epa.gov; Vaituzis.Zigfridas@epamail.epa.gov  
Subject: FW: NOF AF36 includes Efficacy for docket prep/please comment

Shanaz

I have incorporated Peters changes in the Efficacy section (H) of the NOF.

Peter and I have been working back and forth between Word and WordPerfect, so I hope everything comes through formatted ok.

Peter is working on collecting journal articles.

Have a nice weekend.

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
FAX (732)932-8481  
braverman@aesop.rutgers.edu  
IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

From: Mike Braverman [mailto:braverman@aesop.rutgers.edu]  
Sent: Thursday, January 09, 2003 2:34 PM  
To: Bacchus.Shanaz@epamail.epa.gov  
Cc: Peter Cotty; Hutton.Phil@epamail.epa.gov;  
Kough.John@epamail.epa.gov; etsitty.carl@epamail.epa.gov;  
Tomimatsu.Gail@epamail.epa.gov; Vaituzis.Zigfridas@epamail.epa.gov  
Subject: FW: NOF AF36 includes Efficacy for docket prep/please comment

Shanaz

Sorry that I missed one part with USDA. I have corrected the NOF so it all reads on behalf of the AZ-Cotton Research and Protection Council.

Peter - Please address the questions under section H. Efficacy

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
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IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

From: Bacchus.Shanaz@epamail.epa.gov  
[mailto:Bacchus.Shanaz@epamail.epa.gov]  
Sent: Thursday, January 09, 2003 2:17 PM  
To: Mike Braverman  
Cc: Peter Cotty; Hutton.Phil@epamail.epa.gov;  
Kough.John@epamail.epa.gov; etsitty.carl@epamail.epa.gov;  
Tomimatsu.Gail@epamail.epa.gov; Vaituzis.Zigfridas@epamail.epa.gov  
Subject: NOF AF36 includes Efficacy for docket prep/please comment

Attached is the AF36 revised version including efficacy data as requested by John. I lightened up the acute tox data waivers a bit, since it was redundant. Just a few simple questions left in purple. If you all agree on this version, I'll submit it for typesetting by Friday COB. Can you respond by Friday noon?  
(See attached file: NOF Af36 Sec3 for pub 03.wpd)  
Mike, the behalf section was a bit confusing. Are you submitting the petition on Peter's behalf and the Section 3 on behalf of AZ Cotton Council (AZCC)? Or is it all on behalf of AZ CC?

Thanks.  
Sincerely,  
Shanaz Bacchus, Chemist  
USEPA/OPP (Mail Code 7511C)  
Biopesticides and Pollution Prevention Division  
1200 Pennsylvania Ave., N.W.

Washington D.C. 20460

Phone: 703-308-8097

Fax: 703-308-7026



NOF Af36 Sec3 for pub 03.wf



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

01/10/03 04:04 PM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc: Peter Cotty <picotty@srrc.ars.usda.gov>, Phil  
Hutton/DC/USEPA/US@EPA, John Kough/DC/USEPA/US@EPA, Carl  
Etsitty/DC/USEPA/US@EPA, Gail Tomimatsu/DC/USEPA/US@EPA,  
Zigfridas Vaituzis/DC/USEPA/US@EPA  
Subject: FW: NOF AF36 includes Efficacy for docket prep/please comment

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IR-4 Website [www.cook.rutgers.edu/~ir4](http://www.cook.rutgers.edu/~ir4)

-----Original Message-----

From: Mike Braverman (mailto:braverman@aesop.rutgers.edu)  
Sent: Thursday, January 09, 2003 2:34 PM  
To: Bacchus.Shanaz@epamail.epa.gov  
Cc: Peter Cotty; Hutton.Phil@epamail.epa.gov;  
Kough.John@epamail.epa.gov; etsitty.carl@epamail.epa.gov;  
Tomimatsu.Gail@epamail.epa.gov; Vaituzis.Zigfridas@epamail.epa.gov  
Subject: FW: NOF AF36 includes Efficacy for docket prep/please comment

Shanaz

Sorry that I missed one part with USDA. I have corrected the NOF so it all  
reads on behalf of the AZ Cotton Research and Protection Council.

Peter - Please address the questions under section H. Efficacy

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
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braverman@aesop.rutgers.edu

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-----Original Message-----

From: Bacchus.Shanaz@epamail.epa.gov  
[mailto:Bacchus.Shanaz@epamail.epa.gov]  
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Subject: NOF AF36 includes Efficacy for docket prep/please comment

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(See attached file: NOF Af36 Sec3 for pub 03.wpd)  
Mike, the behalf section was a bit confusing. Are you submitting the petition on Peter's behalf and the Section 3 on behalf of AZ Cotton Council (AZCC) ? Or is it all on behalf of AZ CC?

Thanks.

Sincerely,

Shanaz Bacchus, Chemist  
USEPA/OPP (Mail Code 7511C)  
Biopesticides and Pollution Prevention Division  
1200 Pennsylvania Ave., N.W.  
Washington D.C. 20460  
Phone: 703-308-8097  
Fax: 703-308-7026



NOF Af36 Sec3 for pub 03.wpd



Mike Braverman  
<braverman@AESOP.  
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01/09/03 02:34 PM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc: Peter Cotty <pjcott@srcc.ars.usda.gov>, Phil  
Hutton/DC/USEPA/US@EPA, John Kough/DC/USEPA/US@EPA, Carl  
Etsitty/DC/USEPA/US@EPA, Gail Tomimatsu/DC/USEPA/US@EPA,  
Zigfridas Vaituzis/DC/USEPA/US@EPA  
Subject: FW: NOF AF36 includes Efficacy for docket prep/please comment

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NOF Af36 Sec3 for pub 03.wq



Mike Braverman  
<braverman@AESOP.  
RUTGERS.EDU>

01/06/03 03:33 PM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc: Peter Cotty <pcotty@srcc.ars.usda.gov>  
Subject: Efficacy summary in NOF

Shanaz

The NOF is attached with the efficacy summary near the end of the file.

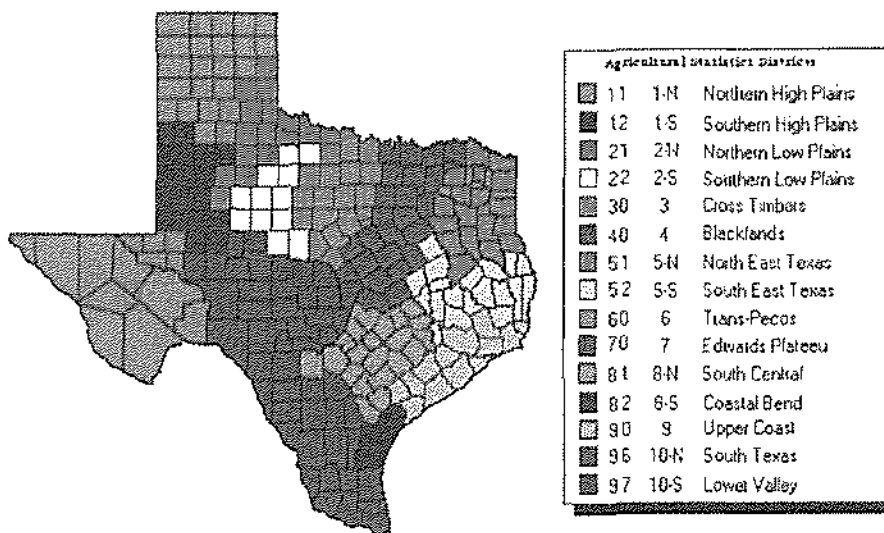
Peter-Thanks

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
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AF36Sec 3NOFNEW2.wj

## Texas Agricultural Statistical Districts



# Texas Irrigated Upland Cotton County Estimates 2001

For Information Contact: Betty Johnson

Link To: District Map

District Code	County FIPS Code	District and County	Acreage Planted (acres)	Acreage Harvested (acres)	Yield per Harvested Acre (pounds)	Production (bales)
11	45	Briscoe	24,000	24,000	530	26,500
	65	Carson	5,000	4,700	735	7,200
	69	Castro	78,000	77,900	955	155,000
	117	Deaf Smith	27,000	23,000	772	37,000
	153	Floyd	125,500	120,600	653	164,000
	189	Hale	242,000	238,500	708	352,000
	205	Hartley	1,400	1,100	655	1,500
	369	Parmer	70,000	69,000	1,043	150,000
	381	Randall	2,000	2,000	600	2,500
	437	Swisher	72,000	69,500	780	113,000
	888	Other Counties	2,100	1,700	649	2,300
	999	District 1-N	649,000	632,000	768	1,011,000
12	3	Andrews	9,000	8,200	585	10,000
	17	Bailey	36,000	29,800	660	41,000
	79	Cochran	71,000	69,000	563	81,000
	107	Crosby	136,000	98,000	451	92,000
	115	Dawson	56,000	52,500	622	68,000
	165	Gaines	184,000	167,500	519	181,000
	173	Glasscock	23,500	23,000	668	32,000
	219	Hockley	139,000	92,000	522	100,000

District Code	County FIPS Code	District and County	Acreage Planted (acres)	Acreage Harvested (acres)	Yield per Harvested Acre (pounds)	Production (bales)
	227	Howard	2,500	2,300	417	2,000
	279	Lamb	163,500	150,500	753	236,000
	303	Lubbock	171,500	131,000	447	122,000
	305	Lynn	80,500	35,800	389	29,000
	317	Martin	10,500	10,400	692	15,000
	329	Midland	8,000	7,000	480	7,000
	445	Terry	125,500	119,000	387	96,000
	501	Yoakum	63,500	61,000	567	72,000
	999	District 1-S	1,280,000	1,057,000	538	1,184,000
21	33	Borden	1,500	1,500	704	2,200
	75	Childress	6,100	6,100	708	9,000
	87	Collingsworth	5,600	5,300	860	9,500
	101	Cottle	1,800	1,800	667	2,500
	125	Dickens	3,100	3,100	325	2,100
	129	Donley	4,700	4,600	522	5,000
	169	Garza	12,200	10,600	543	12,000
	191	Hall	8,200	7,800	677	11,000
	197	Hardeman	3,000	2,800	600	3,500
	345	Motley	2,200	2,000	600	2,500
	483	Wheeler	1,300	1,200	800	2,000
	487	Wilbarger	1,000	900	693	1,300
	888	Other Counties	300	300	640	400
	999	District 2-N	51,000	48,000	630	63,000

District Code	County FIPS Code	District and County	Acreage Planted (acres)	Acreage Harvested (acres)	Yield per Harvested Acre (pounds)	Production (bales)
22	151	Fisher	2,200	2,200	873	4,000
	207	Haskell	16,300	15,800	668	22,000
	275	Knox	16,000	16,000	846	28,200
	335	Mitchell	3,000	3,000	736	4,600
	353	Nolan	2,700	2,500	691	3,600
	399	Runnels	1,400	1,400	514	1,500
	415	Scurry	1,400	1,400	514	1,500
	888	Other Counties	2,000	1,700	734	2,600
	999	District 2-S	45,000	44,000	742	68,000
40	888	Other Counties	2,000	2,000	720	3,000
	999	District 4	2,000	2,000	720	3,000
51	888	Other Counties	1,000	1,000	480	1,000
	999	District 5-N	1,000	1,000	480	1,000
52	41	Brazos	5,400	5,400	800	9,000
	395	Robertson	18,600	18,600	568	22,000
	999	District 5-S	24,000	24,000	620	31,000
60	141	El Paso	9,000	8,700	1,324	24,000
	229	Hudspeth	8,000	8,000	1,200	20,000
	371	Pecos	6,500	6,500	849	11,500
	389	Reeves	5,700	5,200	591	6,400
	888	Other Counties	800	600	880	1,100
	999	District 6	30,000	29,000	1,043	63,000
70	95	Concho	1,600	1,600	600	2,000

District Code	County FIPS Code	District and County	Acreage Planted (acres)	Acreage Harvested (acres)	Yield per Harvested Acre (pounds)	Production (bales)
	383	Reagan	7,400	7,400	584	9,000
	451	Tom Green	12,900	12,900	670	18,000
	461	Upton	5,300	5,300	607	6,700
	888	Other Counties	6,800	6,800	868	12,300
	999	District 7	34,000	34,000	678	48,000
81	25	Bee	1,400	1,400	1,029	3,000
	51	Burleson	7,900	7,900	711	11,700
	888	Other Counties	4,700	4,700	1,256	12,300
	999	District 8-N	14,000	14,000	926	27,000
82	409	San Patricio	7,000	7,000	1,234	18,000
	999	District 8-S	7,000	7,000	1,234	18,000
90	469	Victoria	1,000	1,000	720	1,500
	481	Wharton	5,000	5,000	912	9,500
	888	Other Counties	1,000	1,000	960	2,000
	999	District 9	7,000	7,000	891	13,000
96	507	Zavala	2,800	2,800	857	5,000
	888	Other Counties	2,200	2,200	873	4,000
	999	District 10-N	5,000	5,000	864	9,000
97	61	Cameron	36,800	35,000	617	45,000
	215	Hidalgo	42,500	40,000	780	65,000
	489	Willacy	7,600	5,900	732	9,000
	888	Other Counties	2,100	2,100	686	3,000
	999	District 10-S	89,000	83,000	706	122,000

District Code	County FIPS Code	District and County	Acreage Planted (acres)	Acreage Harvested (acres)	Yield per Harvested Acre (pounds)	Production (bales)
99	999	Texas	2,238,000	1,987,000	643	2,661,000

When less than 1,000 planted acres of dryland or irrigated crop are estimated for a county or district, the acres and production for both practices are included in "other counties" or "other districts" to avoid disclosure.

# Texas Non-Irrigated Upland Cotton County Estimates 2001

For Information Contact: Betty Johnson  
Link to: District Map

District Code	County FIPS Code	District and County	Acreage Planted (acres)	Acreage Harvested (acres)	Yield per Harvested Acre (pounds)	Production (bales)
11	45	Briscoe	17,000	15,400	237	7,600
	65	Carson	2,200	2,100	434	1,900
	69	Castro	3,700	2,400	400	2,000
	117	Deaf Smith	8,000	6,700	394	5,500
	153	Floyd	51,600	47,000	255	25,000
	189	Hale	24,700	22,500	416	19,500
	205	Hartley	5,600	2,200	415	1,900
	369	Parmer	4,400	4,100	433	3,700
	381	Randall	2,200	1,700	424	1,500
	437	Swisher	9,200	9,000	400	7,500
	888	Other Counties	2,400	900	480	900
	999	District 1-N	131,000	114,000	324	77,000
12	3	Andrews	19,100	9,000	107	2,000
	17	Bailey	50,000	40,000	240	20,000
	79	Cochran	61,600	44,500	248	23,000
	107	Crosby	88,400	74,500	161	25,000
	115	Dawson	253,000	32,000	210	14,000
	165	Gaines	108,500	24,000	180	9,000
	173	Glasscock	68,900	7,000	206	3,000

District Code	County FIPS Code	District and County	Acreage Planted (acres)	Acreage Harvested (acres)	Yield per Harvested Acre (pounds)	Production (bales)
	219	Hockley	118,400	73,500	229	35,000
	227	Howard	126,000	23,000	125	6,000
	279	Lamb	42,400	19,500	345	14,000
	303	Lubbock	92,500	62,500	184	24,000
	305	Lynn	219,000	74,500	209	32,500
	317	Martin	146,500	15,000	112	3,500
	329	Midland	26,800	7,000	137	2,000
	445	Terry	140,200	69,500	162	23,500
	501	Yoakum	83,700	26,500	172	9,500
	999	District 1-S	1,645,000	602,000	196	246,000
21	33	Borden	19,700	4,000	180	1,500
	75	Childress	33,300	30,000	176	11,000
	87	Collingsworth	12,500	12,000	268	6,700
	101	Cottle	17,200	12,400	135	3,500
	125	Dickens	21,200	20,000	242	10,100
	129	Donley	8,800	7,600	360	5,700
	169	Garza	32,600	13,500	142	4,000
	191	Hall	49,700	42,800	236	21,000
	197	Hardeman	4,900	2,500	192	1,000
	263	Kent	3,000	2,900	248	1,500
	345	Motley	27,700	25,000	134	7,000
	483	Wheeler	3,500	3,500	274	2,000
	485	Wichita	18,700	8,800	191	3,500

District Code	County FIPS Code	District and County	Acreage Planted (acres)	Acreage Harvested (acres)	Yield per Harvested Acre (pounds)	Production (bales)
	487	Wilbarger	30,000	25,000	161	8,400
	888	Other Counties	16,200	5,000	202	2,100
	999	District 2-N	299,000	215,000	199	89,000
22	83	Coleman	5,000	3,500	192	1,400
	151	Fisher	71,500	64,500	223	30,000
	207	Haskell	79,400	76,000	202	32,000
	275	Knox	13,500	10,500	229	5,000
	335	Mitchell	55,600	40,000	180	15,000
	353	Nolan	53,000	48,000	175	17,500
	399	Runnels	55,900	50,000	206	21,500
	415	Scurry	63,000	51,000	179	19,000
	888	Other Counties	128,100	102,500	214	45,600
	999	District 2-S	525,000	446,000	201	187,000
30	49	Brown	2,200	500	192	200
	77	Clay	1,700	1,000	144	300
	417	Shackelford	2,500	2,400	260	1,300
	447	Throckmorton	3,400	2,500	154	800
	888	Other Counties	2,200	1,600	120	400
	999	District 3	12,000	8,000	180	3,000
40	27	Bell	1,500	1,500	416	1,300
	85	Collin	2,900	2,800	360	2,100
	119	Delta	1,300	1,200	440	1,100
	139	Ellis	35,000	34,200	441	31,400

District Code	County FIPS Code	District and County	Acreage Planted (acres)	Acreage Harvested (acres)	Yield per Harvested Acre (pounds)	Production (bales)
	217	Hill	23,200	23,000	361	17,300
	231	Hunt	3,500	3,400	452	3,200
	277	Lamar	2,900	2,900	364	2,200
	293	Limestone	3,000	3,000	400	2,500
	349	Navarro	17,300	17,000	339	12,000
	491	Williamson	21,800	20,800	508	22,000
	888	Other Counties	20,600	19,200	498	19,900
	999	District 4	133,000	129,000	428	115,000
51	888	Other Counties	7,000	6,000	480	6,000
	999	District 5-N	7,000	6,000	480	6,000
52	41	Brazos	2,400	2,100	480	2,100
	395	Robertson	3,100	2,900	314	1,900
	471	Walker	1,300	1,100	524	1,200
	888	Other Counties	1,200	900	427	800
	999	District 5-S	8,000	7,000	411	6,000
70	95	Concho	27,300	24,500	186	9,500
	307	McCulloch	3,900	3,500	165	1,200
	383	Reagan	30,800	1,200	320	800
	413	Schleicher	7,500	6,000	392	4,900
	451	Tom Green	74,800	66,800	259	36,000
	461	Upton	12,000	400	240	200
	888	Other Counties	4,700	600	320	400
	999	District 7	161,000	103,000	247	53,000

District Code	County FIPS Code	District and County	Acreage Planted (acres)	Acreage Harvested (acres)	Yield per Harvested Acre (pounds)	Production (bales)
81	15	Austin	4,300	2,600	628	3,400
	25	Bee	11,700	11,700	431	10,500
	51	Burleson	3,200	3,100	387	2,500
	55	Caldwell	3,300	3,100	449	2,900
	89	Colorado	6,800	4,100	644	5,500
	175	Goliad	1,500	1,500	480	1,500
	255	Karnes	1,200	1,200	480	1,200
	453	Travis	3,300	3,100	294	1,900
	888	Other Counties	2,700	2,600	480	2,600
	999	District 8-N	38,000	33,000	465	32,000
82	273	Kleberg	43,300	18,000	427	16,000
	355	Nueces	144,600	117,000	570	139,000
	391	Refugio	41,200	40,600	875	74,000
	409	San Patricio	125,000	123,500	591	152,000
	888	Other Counties	900	900	533	1,000
	999	District 8-S	355,000	300,000	611	382,000
90	39	Brazoria	7,700	7,700	692	11,100
	57	Calhoun	27,500	25,800	683	36,700
	239	Jackson	35,500	30,500	738	46,900
	469	Victoria	18,300	15,700	605	19,800
	481	Wharton	74,000	70,500	783	115,000
	888	Other Counties	95,000	90,800	732	138,500
	999	District 9	258,000	241,000	733	368,000

District Code	County FIPS Code	District and County	Acreage Planted (acres)	Acreage Harvested (acres)	Yield per Harvested Acre (pounds)	Production (bales)
96	131	Duval	1,500	900	480	900
	888	Other Counties	20,500	17,100	396	14,100
	999	District 10-N	22,000	18,000	400	15,000
97	61	Cameron	44,600	13,000	247	6,700
	215	Hidalgo	42,500	8,500	282	5,000
	489	Willacy	80,000	19,000	202	8,000
	888	Other Counties	900	500	288	300
	999	District 10-S	168,000	41,000	234	20,000
99	999	Texas	3,762,000	2,263,000	339	1,599,000

When less than 1,000 planted acres of dryland or irrigated crop are estimated for a county or district, the acres and production for both practices are included in "other counties" or "other districts" to avoid disclosure.

Further Comments on the Efficacy of *Aspergillus flavus* AF36 in Response to Questions received March 10, 2003, from USSEPA, OPP, Biopesticides and Pollution Prevention Division

Peter J. Cotty, Ph.D., Research Plant Pathologist, USDA, ARS, SRRC, New Orleans, LA 70124. Phone: 504-286-4391

Michael Braverman, Ph.D., Biopesticide Manager, IR-4, Rutgers University, New Brunswick, NJ 08902. Phone: 732-932-9575

Applications of *Aspergillus flavus* AF36 seek to alter the *A. flavus* communities resident in agricultural fields so that the non-aflatoxin (atoxicogenic) strain AF36 is more common and highly toxigenic strains (such as the S strain) are less common. This results in reductions in the average aflatoxin producing potential of *A. flavus* communities associated with treated crops and resident in treated fields.

These are the activities we claim for the product *Aspergillus flavus* AF36. **We do not claim to reduce aflatoxin content to any given level.** In some areas and years, aflatoxin content may exceed 2,000 ppb in the seed and a fairly successful displacement (80%) would only be expected to achieve a reduction to a level in excess of 400 ppb. Yet, in many cases, the industry and particularly the producer living on the farm, would view this as advantageous.

Aflatoxin contamination of cottonseed is monitored in several ways in different areas. In general, it is carefully monitored going into dairy markets. The FDA does not perform this monitoring, although they may do spot checks. Industry performs the analyses. Aflatoxin content of the milk is often monitored carefully and if toxin is detected (at 0.3 ppb) the dairies begin looking for the source (usually corn, cottonseed, or milo). If toxin exceeds 0.5 ppb, the milk must be dumped and the dairy is placed on quarantine. The liability for this generally lies on the provider(s) of the feed.

The FDA has different action levels for cottonseed going into different markets:

- Cottonseed may only contain 20 ppb to be used for dairy cattle.
- Cottonseed containing up to 300 ppb can be fed at beef feedlots (i.e. for finishing cattle).
- Cottonseed meal intended for beef cattle, swine or poultry may contain up to 300 ppb aflatoxin.

Even cottonseed exceeding 300 ppb often has markets. It may be sold to an oil mill where the crush must be carefully monitored to maintain meal below 300 ppb. This seed may be sold to cottonseed brokers that ammoniate the contaminated seed to reduce contamination or it may be sold to markets where vegetable proteins are so highly valued that process methods for dealing with aflatoxin contaminated seeds have been developed (i.e. certain Mexican markets).

The aflatoxin content of each lot of seed sold into these markets is generally known and identified. The quantity of aflatoxins influences the value of the seed. It is more difficult to ammoniate seed that exceeds 2,000 ppb than seed that is only 400 ppb. Seed with lower aflatoxin may be more valuable in secondary markets such as Mexico. Seed with lower aflatoxin is more likely to produce meal with aflatoxin contents acceptable for some uses.

It is not unusual for aflatoxin contents to vary by several orders of magnitude between adjacent fields and across adjacent years. Thus it is not feasible to assess the impact of applications directly on aflatoxin contents. Instead we rely on measurements of successful displacement and on the experience of participating gins and producers. Typically, initial areas to be treated are those that have the severe problems with contamination. An example of this was the first farm we treated in 1996 that had 7,000 ppb the previous year. This selection of fields to participate by producers and gins further complicates the toxin view. Nevertheless, we can and have accurately measured displacement of aflatoxin producers and increases in the incidence of the non-aflatoxin producing AF36 on crops and in soils through both the use of repeated measures tests and analysis of variance in replicated trials. The relationship of this displacement to reductions in contamination has been proven in laboratory, greenhouse, and field-plot tests. In commercial field tests, models using cottonseed oil free fatty acid content as a measure of weathering have also supported this relationship. See report entitled "Report on Results of Experimental Program on the use of Atoxigenic *Aspergillus flavus* strain AF36 on Cotton Performed Under Experimental Use Permit 69224-EUP-1: Influences Applications on Communities of *A. flavus* Resident in the Soil of Treated Fields and Assessment of Stability of the Atoxigenic Phenotype of *Aspergillus flavus*" (no MRID assigned). Efficacy data can also be found in MRID 43763405 Cotty, P. Hartman, C. (1995) *Aspergillus flavus* Isolate AF36: Product Performance Data.

We are concerned over potential delays in reviewing the newly requested data. We are open to other ways in which to bring this review to a conclusion. While we do not view this as a public health pesticide we can also amend the label to remove the statements pertaining to reductions in aflatoxin and change the label claims only to include displacement of Aflatoxin producing strains of *Aspergillus flavus*. By removing the claim for reducing aflatoxin, AF36 should certainly not be considered a public health pesticide so there is no need to review the efficacy data and the review can be brought to a conclusion. If efficacy is reviewed, it should be based on the reduction of toxigenic strains.

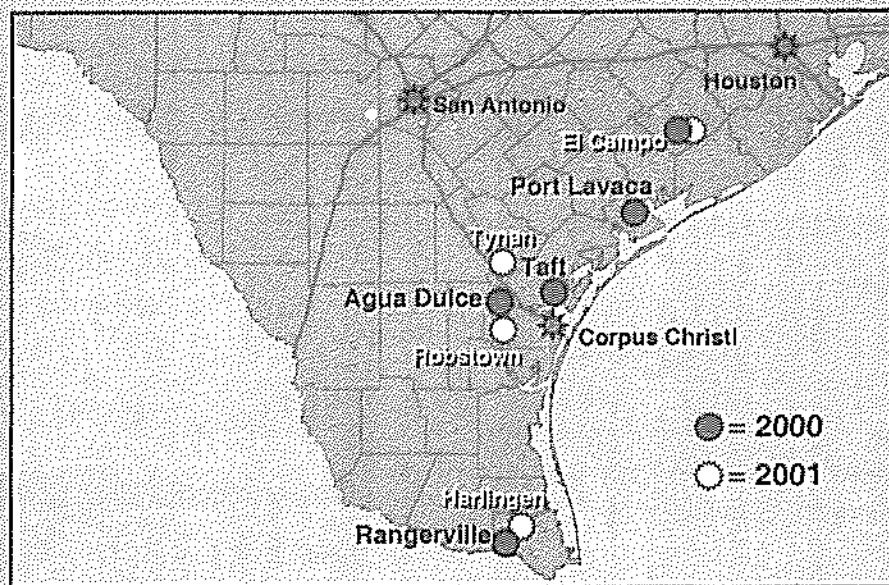
The above mentioned report provides extensive evidence for the efficacy of *Aspergillus flavus* AF-36 in reducing the proportion of the *A. flavus* community composed of the S strain. The S strain produces very high aflatoxin quantities and is a very significant component of the *A. flavus* community in both Arizona and South Texas. Information on the efficacy of AF-36 in modifying *A. flavus* communities in Texas follows.

Efficacy of *Aspergillus flavus* AF36 in Texas: Results of Field Tests on the 2000 and 2001 Commercial Cottonseed Crops.

Peter J. Cotty  
Research Plant Pathologist  
USDA-ARS-SRRC  
P.O. Box 19687, New Orleans, LA 70124  
504-286-4391

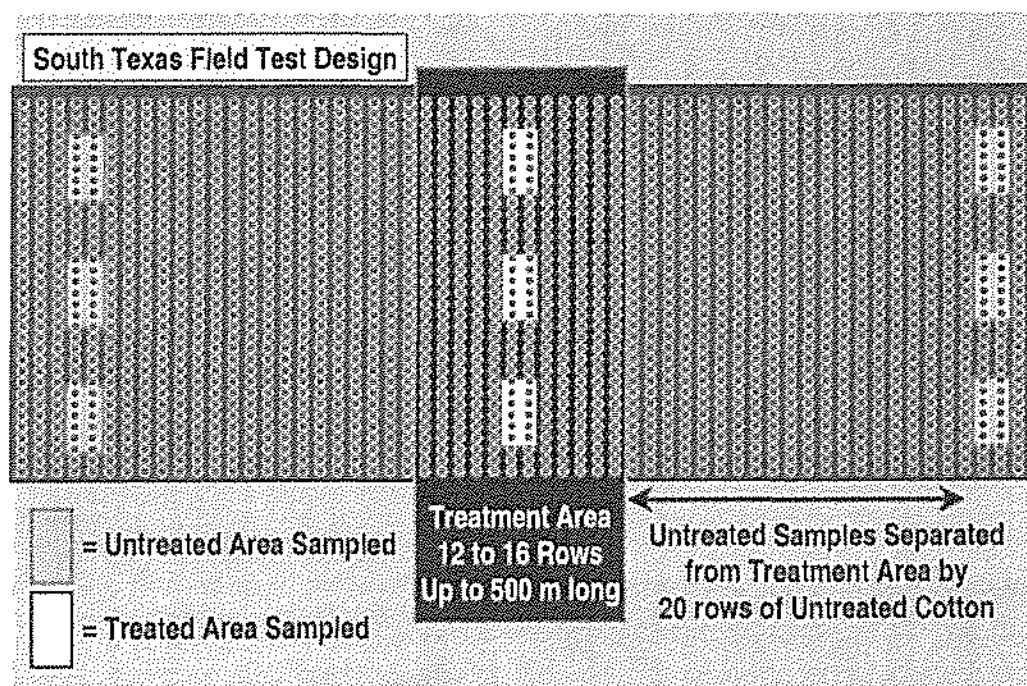
In order to assess efficacy of soil applied *Aspergillus flavus* AF36 in South Texas, field tests were performed in commercial cotton fields at 9 locations throughout South Texas. Tests extended from Rangerville in the Lower Rio Grande Valley area to El Campo in the Upper Coast area.

Locations of South Texas Field Tests in 2000 and 2001



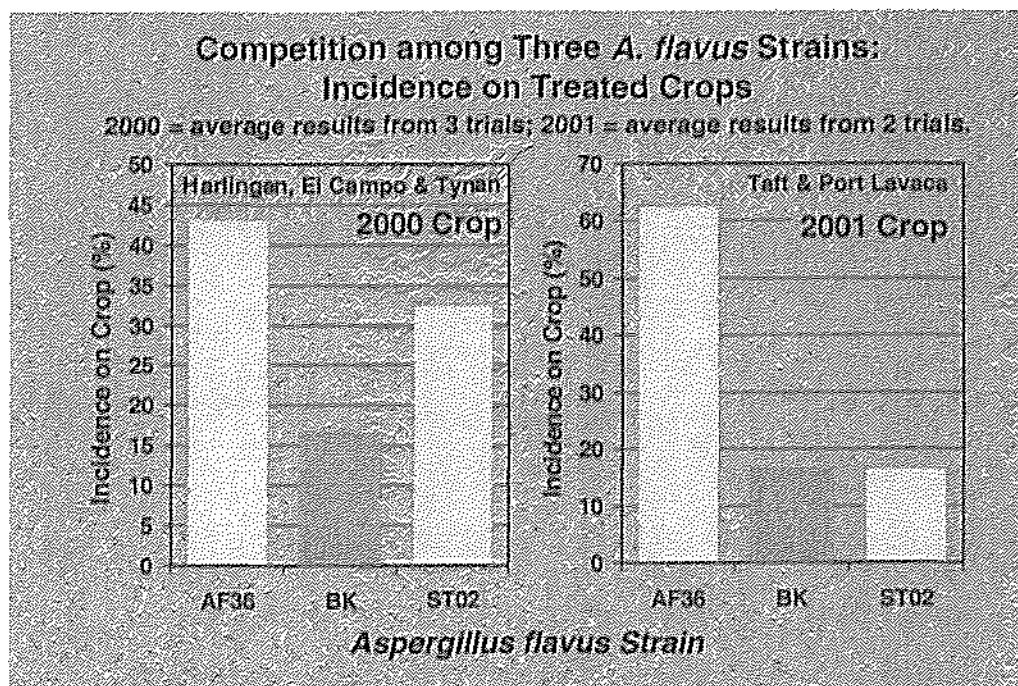
For each test from 0.5 to 1.0 acre of cotton was treated by hand by sprinkling the standard wheat seed formulation of *Aspergillus flavus* AF36 on the soil at the standard rate of 10 lb./acre. In all tests multiple atoxigenic strains of *A. flavus* native to South Texas were evaluated. All strains were applied to the same area at the same rate in order to observe competition among strains and differences among strains in efficacy. Efficacy of atoxigenic strains at displacing the highly toxigenic S strain and other native strains was assessed by characterizing the communities of fungi associated with the mature crop in both treated areas and

in untreated control areas separated from the treated areas by 20 rows of untreated cotton (see field test design below).

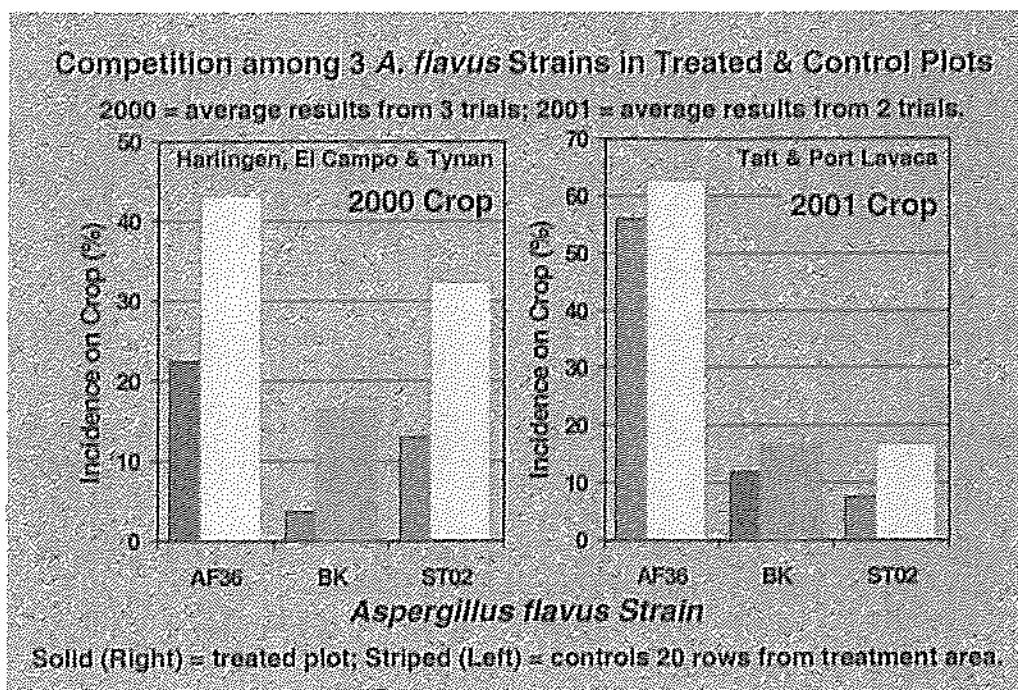


Long-term influences of atoxigenic strains of *A. flavus* have been observed in Arizona. Comparing the communities of fungi resident in the soil just prior to treatment with the community present one year after treatment typically is used to assess this. Such comparisons determine if influences of treatments can be expected to provide a benefit to the environment and crops the second year by reducing the average aflatoxin producing potential of fungi resident in the field across multiple years. The potential for long-term influences of atoxigenic strain applications in South Texas was determined by analyzing the composition of the *A. flavus* communities in the soil of treated plots prior to treatment, with the community structures one year after treatment. Similar comparisons were made contrasting soil in the untreated control plots.

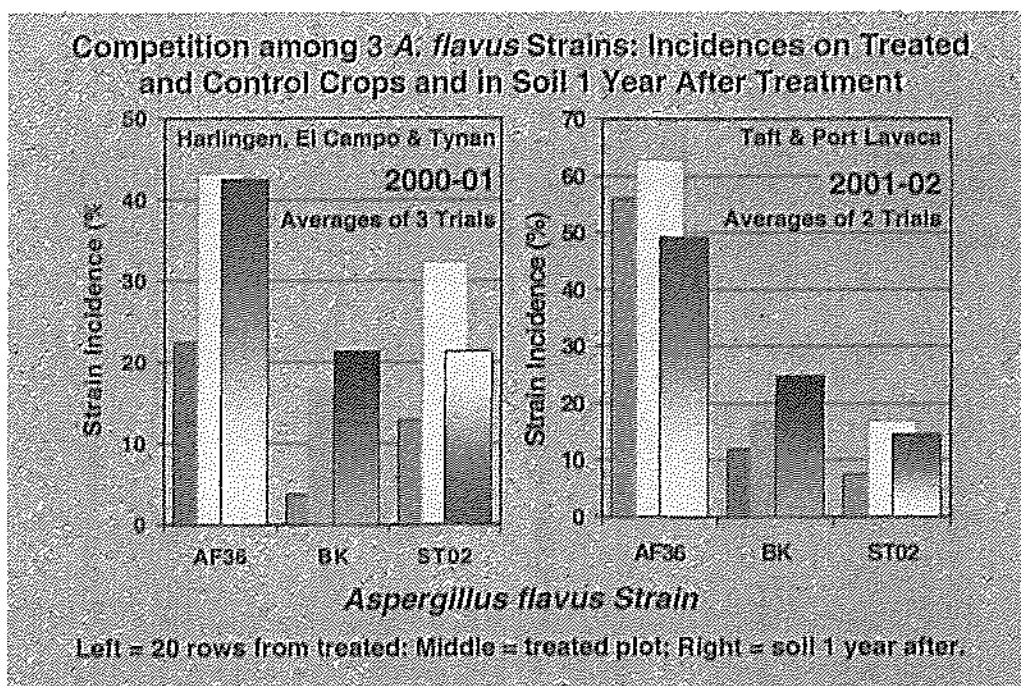
In these studies, three atoxigenic strains (AF36, BK, and ST02) were applied to the treatment area. Incidence of AF36 on treated crops in 2000 and 2001 demonstrate efficacy of *Aspergillus flavus* AF36 at displacing aflatoxin producers during crop colonization.



Both AF36 and the other two-atoxic strains evaluated were effective at spreading from the applied product to the crop and displacing aflatoxin producers during the process. Each strain was applied a single time at 10 pounds per acre. AF36 was the most effective strain in these tests. All strains had efficacy in displacing resident aflatoxin producers.

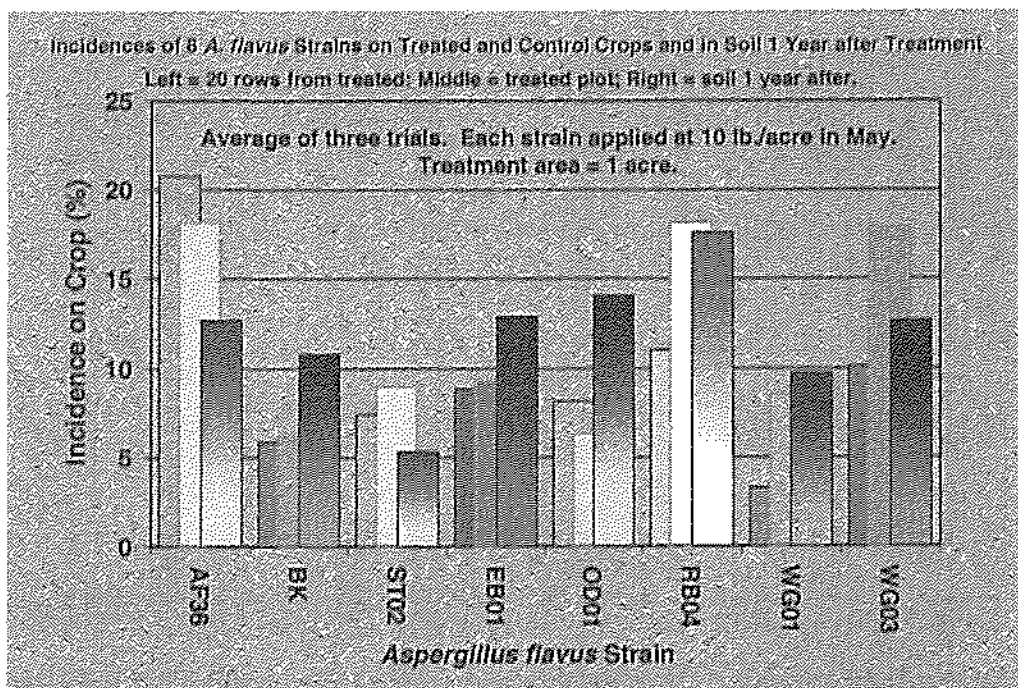


In all tests, AF36 also demonstrated the ability to spread within treated fields across untreated areas. This activity has repeatedly been observed in Arizona and is an aspect of the efficacy of AF36 in displacing aflatoxin producers. The goal of AF36 applications is to modify *A. flavus* communities so that they have a lower potential to produce aflatoxins. The tests in Texas demonstrate great efficacy of AF36 in achieving that goal.

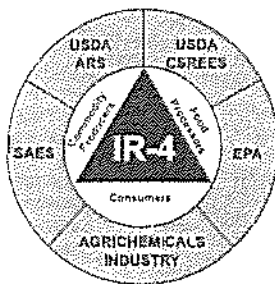


An important aspect of the efficacy of AF36 in Arizona is the ability of applications to make changes to the composition of *A. flavus* communities in soils that are detectable even the season after application. This allows the possibility of inducing long-term reductions in the aflatoxin-producing potential of *A. flavus* communities resident in fields and thus provides the potential to get additive reductions over time. This allows for long-term reductions in the quantity of aflatoxins in crops and in the environment.

Tests performed in Texas in 2000 and 2001 demonstrated excellent efficacy in producing long-term influences of atoxigenic strain applications similar to those seen in Arizona.



Three tests in 2001 were also performed in which 8 atoxigenic strains were compared for efficacy in ability to competitively exclude aflatoxin producers. *Aspergillus flavus* AF36 demonstrated superior efficacy in these trials as well.



**Interregional Research Project No. 4  
Center for Minor Crop Pest Management**

Dr. Janet Andersen  
Biopesticide and Pollution Prevention Division  
Environmental Protection Agency  
Room 910, Crystal Mall 2  
1921 Jefferson Davis Highway  
Arlington, Virginia  
(703) 308-8712

November 12, 2002

RE: *Aspergillus flavus* AF36 8E5001

Minutes of meeting-Section 3 Registration Update: November 7, 2002

EPA Participants- Shanaz Bacchus, John Kough, Zigfridas Vaituzis

Registrant Related Participants- Peter Cotty-USDA/ARS, Phil Wakelyn- National Cotton Council, Larry Antilla- Arizona Cotton Council, Michael Braverman-IR-4 Project.

Dear Janet:

On behalf of the USDA/ARS, National Cotton Council, Arizona Cotton Research and Protection Council and the IR-4 Project, thanks to your staff for taking the time to meet with us on November 7, 2002 to discuss the section 3 registration of *Aspergillus flavus* AF-36.

The most recent previous Section 3 meeting was held on May 30, 2002 (minutes attached). From the previous meeting it was agreed that the studies needed to complete the registration package were the toxicology studies, which have now been submitted. It was also agreed that some of the current EUP label language was due to the lack of toxicology data at the time the label was approved. The goal for completion of the section 3 registration was stated as February.

The most important points to draw from the discussion on November 7<sup>th</sup> are as follows:

1. The strong desire of both parties was clearly expressed that the section 3 should be completed in time to avoid the need for another EUP.
2. A minimum of 40,000 acres was needed for 2003 through a Section 3, conditional registration or EUP or we are in danger of losing this technology.

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Phil Wakelyn began the meeting by highlighting the importance of this project to the cotton industry and how widespread the aflatoxin problem was in 2002 for all crops. Despite over 100 million dollars having been invested in aflatoxin research since 1990 there have been no effective control measures developed except for atoxigenic strain (AF-36) technology. Cotty commented that in addition to cottonseed problems in 2002, there were severe problems in corn and this extended into the mid-west as far as Nebraska as well as devastating the corn crop in South Texas. Larry Antilla described the dire nature of the project and that delay in registration had eroded grower confidence in the program's potential. The lack of a section 3 registration in time for the 2003 could mean the lack of grower involvement and inadequate financial support for the production facility. A minimum of 40,000 acres is needed to keep the operation functional and to keep the early adopters of the program.

Michael Braverman mentioned that all the health effects and ecotox data had been submitted and mentioned the studies involved. Shanaz and Michael had met earlier in the day to discuss the organization of the submission and cleared up questions about some of the signature issues with Teresa Downs. MRID numbers have now been assigned to the previously rejected volumes. The MRID numbers could also be used in developing and submitting an Index of Documents. Michael Braverman questioned how the review time would be influenced by an internal review versus a contract reviewer. It was stated that the turn around time for contract reviewers was 6 weeks and the ecological effects studies were going out that day. The need to still reformat some of the data waiver requests was also discussed and Zigfridas Varnizis mentioned the need to better address the Ecological Effects data waivers, more specifically estuarine, marine environments and daphnia. He also mentioned the need to explain why the avian study was performed using intratracheal instillation and questioned the applicability of the compliance statement to U.S. GLP's.

Throughout the discussion the strong desire of both parties was clearly expressed that the section 3 should be completed to avoid the need for another EUP. Another EUP would unnecessarily use the resources of all parties that are needed to be directed to getting the Section 3 finished in a timely manner. Considering the current EUP was submitted in January of 2002 and was not approved until July, 2002 (since treatments need to be made in May, it was not possible to make treatments in Texas in 2002) there was concern that a delay in submitting an EUP to cover at least 40,000 acres in Arizona may result in no change for the 2003 season.

Label language was discussed in detail. Addition of 2,000 acres in South Texas had resulted in what appeared to be disproportionately large changes to the label language. Some of the language had become much more restrictive and confusing. The meanings of some of the label language was unclear and EPA participants assured the industry representatives that they would provide an interpretation for the questioned portions. It was agreed that it should be possible to amend the label for the 2003 season even without a new EUP.

The overall desire to complete the Section 3 rather than submit another EUP was reiterated but tempered with the concern over the disruption that would be caused if the Section 3 were not complete. The general feeling of the Arizona Cotton Research and Protection Council was guarded optimism based on a sincere effort expressed by BPPD to complete the section 3 by February. Consequently the decision to proceed with an EUP will depend on reasonable progress on the section 3 coupled with greater firmness in the proposed completion date as the review of the toxicology data progresses. Shanaz Bacchus indicated that the toxicology reports would be sent to an external reviewer that day (November 7) and that typical time for completing the review time was 6 weeks.

The potential for a conditional registration was also discussed. It was agreed that if uncertainty developed about completion of data reviews with sufficient time to complete the Section 3 for the 2003

crop treatments, a conditional registration could alleviate the need for an EUP. The bottom line in this discussion was that it is essential that a useable registration be in place for the 2003 season (i.e. by Mar-Apr 2003). A complete Section 3 registration is preferred. However, a conditional registration or modified EUP could serve the 2003 needs.

Although a Section 3 is desired, the timetable for its completion was not specifically mentioned by BPPD. Considering this, answers to the following questions are needed in order to proceed.

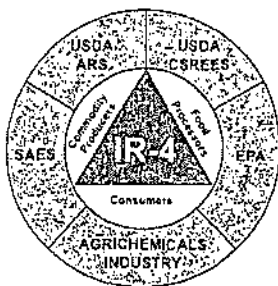
1. If a section 3 registration completion date can not be firmed in the near future, at what point (what date ?) does the decision to move forward with a conditional registration need to be made to allow for a label enabling the use of AF-36 under a conditional label in May 2003?
2. If a section 3 registration completion date can not be firmed in the near future, and it is decided that a conditional registration is not an option, at what point (what date ?) does an expanded EUP need to be submitted to allow for a label enabling the use of AF-36 on at least 40,000 acres in Arizona in May 2003.

Sincerely ,



Michael Braverman  
Biopesticide Coordinator  
IR-4 Project

CC: Phil Hutton, Shanaz Bacchus, John Kough, Zigfridass Vaituzis, Peter Cotty, Phil Wakelyn, Larry Antilla, Bob Holm, Jerry Baron, Dan Kunkel



**Interregional Research Project No. 4  
Center for Minor Crop Pest Management**

Dr. Janet Andersen  
Biopesticide and Pollution Prevention Division  
Environmental Protection Agency  
Room 910, Crystal Mall 2  
1921 Jefferson Davis Highway  
Arlington, Virginia

June 7, 2002

RE: *Aspergillus flavus* AF36 PC Code 006456  
Minutes of meeting- Section 3 Registration May 30, 2002

EPA Participants- Janet Andersen, Phil Hutton, Shanaz Bacchus, Gail Tomimatsu,  
Zigfridass Vaituzis, Carl Etsitty

Registrant Related Participants- Peter Cotty-USDA/ARS, Phil Wakelyn, Keith Menchey-  
National Cotton Council, Larry Antilla- Arizona Cotton Research and Protection Council,  
Chuck Youngker, Arizona Cotton Growers Association, Michael Braverman-IR-4 Project.

Dear Janet:

On behalf of the USDA/ARS, National Cotton Council, Arizona Cotton Council, Arizona Cotton Growers Association and the IR-4 Project, I would like to thank you and your staff for taking the time to meet with us to discuss the section 3 registration of *Aspergillus flavus* AF-36 on May 30, 2002. We were especially impressed with your interest and questions about the project and its importance to growers and public health. According to my records, the last Section 3 meeting was held about two years ago on June 27, 2000 (copy attached). It appears that from that previous meeting it was agreed that the studies needed to complete the registration package were the toxicology studies, which have now been completed.

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The most important highlights of the current meeting and our understanding of what is needed to complete the data requirements for Section 3 registration for AF-36 and the timetable for EPA review of these data are as follows:

*When formally submitted (and assuming they are acceptable), the mammalian, avian and bee toxicology studies will essentially complete the data requirements for the section 3 registration.*

*Many parts of the current label language were developed due to the lack of toxicology data and if justified, can be modified.*

*The goal for section 3 registration is February 2003 so that we can avoid the inefficiency of having to request and review an additional expansion of the EUP.*

The following are our minutes from the current meeting.

Phil Wakelyn made some opening comments about the importance of this project to cotton growers and that this was a grassroots effort, made up of direct interactions among growers, gins and public agencies. The product is manufactured by the Arizona Cotton Research and Protection Council (a component of the Arizona Department of Agriculture) and distributed directly to growers. He also highlighted the fact that there are no chemical alternatives to aflatoxin management and the agricultural industry welcomed an effective biopesticide solution. EPA appreciated the innovative approach and direct grower involvement.

Larry Antilla talked about the building of the production facility in Arizona over the last 3 years and that about 46,000 acres had been treated. Case studies of aflatoxin reduction figures on several farms were reviewed. One farm with approximately 1,000 acres of cotton had previously never been able to produce cottonseed below 20 ppb. In 2000, that farm produced seed with acceptable aflatoxin contents (<20 ppb) on 82% of its 17 treated fields and in 2001, 86% of treated fields produced cottonseed with acceptable aflatoxin contents. Cluck Younger gave a personal perspective on the aflatoxin problem in Arizona and noted that growers have committed \$2.4 million dollars to the research program. In addition, it was pointed out that the only means of mitigation for high aflatoxin levels in seed involves the injection of anhydrous ammonia, which is caustic and poses human health risks.

There was a general discussion of the toxicology data (which was unofficially submitted in early May). It was generally agreed that the Tween 80 used in the first mammalian study was responsible for some effects that were not related to AF36 which was confirmed by the second study. The toxicology data constitutes the remaining portion of the registration package to be submitted and must be officially submitted before being considered.

Many parts of the current label language were developed due to the lack of toxicology data and if justified, can be modified. The combination of acceptable toxicology data, the lack of an increase in total *Aspergillus* and a reduction in aflatoxin producing spores all contribute to a favorable risk profile due to no change in exposure combined with reduced hazard of AF-36 the spores to the environment. Some of the more specific parts to be considered included adding a

statement that AF36 can be applied to irrigated fields, removal of the buffer statement, wind direction statement, modification of the 12 month storage statement, statement related to inducing vomiting, and perhaps some others.

There was general discussion about the distribution of the S strain of *A. flavus* with regard to areas that the section 3 registration would include. Initially the registration would cover Arizona and Texas.

In the final topic of discussion Phil Hutton questioned the need for a genetic marker test. He deferred this to John Kough (one of the science reviewers not at the meeting). Peter Cotty reviewed comments and data previously submitted on the reliability of the vegetative compatibility test and the stability of AF36 as a genetic group and its frequent occurrence in the environment and explained the history and reliability of the vegetative compatibility method. He indicated that redundant Vegetative Compatibility Testing (VC testing) was a component of the quality control procedures previously submitted and used in the manufacture of *Aspergillus flavus* AF36. Personnel are readily trained to perform VC testing and thousands of such analyses are performed annually in order to assess efficacy of AF36 treatments. A DNA based technique could not be practically applied in as robust a manner. Janet Andersen mentioned that a lot of additional knowledge had been collected since the time the genetic marker test was suggested and that the redundancy of the QA/QC was probably adequate. Janet also suggested that we try to submit most of the information in electronic format in addition to the hard copies to facilitate review.

Now it is up to us to get all of the data in for review so that our goal of section 3 registration by February 2003 can be achieved. While we were previously unaware that this was classified as a public health issue we assume that should add weight to the benefits consideration and prioritize its importance in the review process.

Sincerely,



Michael Braverman, Ph.D  
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IR-4 Project, Rutgers University  
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North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
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braverman@aesop.rutgers.edu

cc: Phil Hutton, Shanaz Bacchus, Gail Tomimatsu, Zigfridass Vaituzis, Carl Etsitty, John Kough  
Peter Cotty, Phil Wakelyn, Keith Menchey, Larry Antilla, Chuck Youngker, Bob Holm



United States  
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Agricultural  
Research  
Service

Mid South Area  
Southern Regional  
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70179-0687

June 23, 2000

Ms. Shanaz Bacchus  
Regulatory Action Leader  
USEPA, BPPD/OPP (7511C)  
Crystal Mall #2, Room 902  
1921 Jefferson Davis Highway  
Arlington, VA 22202  
Phone: 703-308-8097  
FAX: 703-308-7026

Dear Ms. Bacchus:

Dr. W. L. Biehn of the IR-4 project asked me to send a copy of the enclosed manuscript by courier for delivery on Monday June 26<sup>th</sup>. This manuscript is a preliminary draft that we hope to refine for submission for publication in the next few months. I will discuss aspects of the studies covered by the manuscript in our meeting on June 27<sup>th</sup>. I look forward to our discussions on Tuesday.  
Best Regards.

Sincerely,

Peter J. Cotty  
Research Plant Pathologist  
Food and Feed Safety Research

ENCLOSURE

AGENDA

EPA PREREGISTRATION MEETING

MICROBIAL PRODUCT: ASPERGILLUS FLAVUS AF36 ON COTTON IN ARIZONA

Tuesday June 27, 2000 at ~~10:30 AM~~ 1:00 PM *WFB*  
Room 912  
EPA Crystal Mall #2  
1921 Jefferson Davis Highway, Arlington, VA

1. Introductions  
Dr. Phillip J. Wakelyn
2. Airborne spore counts of Aspergillus flavus in treated and untreated fields  
Dr. Peter Cotty
3. Aspergillus flavus in Natural Habitats  
Dr. Peter Cotty
4. Toxicity testing and other data needed for registration
  - a) Acute Pulmonary Toxicity/Pathogenicity Requirement in Mammals
  - b) Avian Acute Oral Toxicity/Pathogenicity Requirement
  - c) Avian Acute Pulmonary Toxicity/Pathogenicity Requirement
  - d) Honey Bee Testing Requirement
5. Other items
  - a) Options for use and testing of AF36 in the year 2001 in Arizona
  - b) Discussion

Seasonal changes in the quantities of *Aspergillus flavus* and other propagules in the air over  
Arizona cotton fields

<sup>1</sup>C.H. Bock and P.J. Cotty

USDA-ARS-SRRC, 1100 Robert E. Lee Boulevard, New Orleans, LA 70124. <sup>1</sup>Current address  
of first author: Centre for Plant Biodiversity Research, CSIRO-Plant Industry, GPO 1600,  
Canberra, ACT 2610, Australia.

# ABSTRACT

Airborne *Aspergillus flavus* was monitored continuously with two Burkard cyclone samplers  
from May 1997 to March 1999 at two sites in Arizona surrounded by commercial agriculture.  
One sampler was initially at the center of 65 ha of cotton treated June 2, 1997 with an atoxigenic  
vegetative compatibility group (VCG) of *A. flavus* and the second sampler was about 1 km from  
the treated field. Both fields went through typical crop rotations. Propagules in the size range of  
conidia were collected by the cyclones at levels comparable to those collected by impaction onto  
0.8  $\mu$ m pore membranes. Quantities of both total fungi and *A. flavus* did not differ ( $P=0.05$ )  
between the two sites. Total fungal propagules ranged from 17 to 667  $\text{m}^{-3}$  and from 9 to 1,277  
 $\text{m}^{-3}$ , at the non-treated and treated sites respectively. Counts of *A. flavus* ranged from <1 to 406  
 $\text{m}^{-3}$  and <1 to 416  $\text{m}^{-3}$  and *A. flavus* comprised 1 to 46% and <1 to 51% of the total cultured  
fungi at the two sites, respectively. Peaks in both total fungal propagules and *A. flavus*  
propagules coincided with area-wide boll maturation and cotton harvest (Julian day 251-321).  
The S strain was most frequent between May and August. In other months, the L strain  
accounted for up to 100% of the *A. flavus* sampled. The applied VCG accounted for 0-47% of  
the L strain at the untreated site and 5-75% at the treated site. The applied VCG was a greater  
proportion of *A. flavus* at the treated site than at the non-treated site ( $P=0.01$ ). Modified rotorod  
samplers collected viable *A. niger*, but little *A. flavus*. Large quantities of *A. flavus* occurred in  
the soil (up to 34,474 propagules  $\text{g}^{-1}$ ) of cotton fields and on cotton plant parts and debris (up to

272,461 propagules g<sup>-1</sup>) adjacent to the cyclone samplers. These data suggest that *A. flavus* is a major constituent of the air associated with cotton fields in southwest Arizona at certain times of the year. Although application of atoxigenic *A. flavus* altered the proportion of strains and VCGs in the aerial mycoflora, it did not alter the total quantity of *A. flavus*. Dispersal of *A. flavus* between fields suggests atoxigenic fungi will be most effective in area-wide management programs.

*Additional keywords:* aflatoxin management; agricultural dust.

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*Aspergillus flavus* Link can infect cottonseed and other food and feed crops under specific environmental conditions (Deiner *et al.*, 1987). Concern for *A. flavus* infections largely stems from fungal production of aflatoxins, potent natural carcinogens. To minimize the risk of toxin carryover to man or livestock, crop aflatoxin content is strictly regulated in many countries (Park *et al.*, 1988).

One approach to limit aflatoxin contamination in cottonseed is application of strains of *A. flavus* that do not produce aflatoxins (atoxigenic strains) to competitively exclude the aflatoxin producing strains (Cotty, 1992; 1993; Cotty, 1994a). One atoxigenic strain is being tested under an EPA approved experimental use permit (69224-EUP-1) on farms in southwest Arizona to assess efficacy in controlling aflatoxin contamination of cottonseed in field scale systems. Atoxigenic strains of *A. flavus* have been effective in experimental plots with peanut (Dorner *et al.*, 1992), corn (Brown *et al.*, 1991, Dorner, 1999), and cotton (Cotty, 1994a).

Although aflatoxin contamination is reduced by application of atoxigenic strains (Cotty, 1994a), there is no basis on which to gauge the overall effect of treatments on quantities of *A. flavus* propagules in the air. Furthermore, the seasonal dynamics of airborne *A. flavus* in cotton production in southwestern Arizona has been incompletely described. Atoxigenic strain applications seek to competitively exclude aflatoxin producers and optimal application of the biocontrol agent is dependent on the community dynamics of aflatoxin-producing *A. flavus*

1 strains. Lack of information on seasonal dynamics of air-borne conidia of *A. flavus* precludes a  
2 rational approach to delimiting optimal periods over which fields may be treated. Dispersal of  
3 an atoxigenic strain prior to the presence of aflatoxin-producers in the air flora will give the  
4 biocontrol agent preferential exposure to potential food sources and thereby increase its  
5 biocompetitive potential.

6 In general, there is little information on seasonal changes in aerially dispersed *A. flavus*  
7 propagules in agriculture. Epidemiological studies have investigated influences of factors on  
8 infection of and aflatoxin formation in cottonseed, (Ashworth *et al.*, 1969a; Ashworth *et al.*,  
9 1969b; Ashworth *et al.*, 1971; Marsh *et al.*, 1973; Diener *et al.*, 1987; Lee *et al.*, 1989) and the  
10 production and dispersal of propagules at specific times in cotton (Lee *et al.*, 1986) or in other  
11 crops (Bothast *et al.*, 1978; Holtmeyer and Wallin, 1980; Holtmeyer and Wallin, 1981; Olanya *et*  
12 *al.*, 1997). Peaks in quantities of *A. flavus* are found in summer months, but previous studies  
13 have not described annual cycles of *A. flavus* in the air spora. The quantity of *A. flavus* in the air  
14 in relation to other fungal propagules in cultivated desert environments of southwest Arizona is  
15 also unknown, although *A. flavus* is a major constituent of the airspora in other environments  
16 (Morrow *et al.*, 1964). Dynamics of interactions among air-borne fungi have not been  
17 investigated.

18 In order to expand available information on characteristics of production and dispersal of  
19 conidia of *A. flavus* in southwest Arizona we sampled air for microbiological propagules for two  
20 years at two sites approximately 1 km apart. Fields surrounding one site were initially planted  
21 with cotton and treated with an atoxigenic strain of *A. flavus*. Fields surrounding the second site  
22 were not treated and initially planted to barley. Information on fungal dynamics of value in  
23 developing application strategies for the aflatoxin biocontrol agent in cotton-producing regions  
24 of Arizona were sought.

## MATERIALS AND METHODS

Cyclone samplers. Two Burkard Cyclone Samplers (Burkard Manufacturing Co. Ltd., Rickmansworth, Hertfordshire, England) were operated continuously from May 1997 to March 1999 (Fig. 1). One sampler was initially in the center of 65 ha of cotton treated with atoxigenic *A. flavus* (strain AF36; Cotty and Bayman, 1993) in 1997 (June 2). The field (16 ha) southeast of the sampler was also treated in 1996. The second sampler was about 1 km from the nearest treated field (Fig. 2). Fields surrounding the samplers remained in commercial agriculture and were rotated through several crops (Table 1).

The Burkard cyclone sampler operates by drawing air through an orifice at a rate of 16.5 L min<sup>-1</sup> and depositing the particles in still air in a microfuge tube at the base of the cyclone stream. Sampler performance was monitored with a hot wire anemometer (Model HHF51, Omega Engineering, Stamford, CT) to ensure air was drawn at the correct rate. Particle size sampling efficiency has been characterized (Emberlin and Baboonian, 1995) and is excellent for particles with the size range of *A. flavus* spores (3-10 µm). In this study samples were taken weekly and each sample was weighed, suspended in sterile distilled water, dilution plated on agar media, and incubated at 31 C. Total colony forming units (CFUs) of all fungi and the number of *A. niger* colony forming units (CFUs) were counted on PDA and 5/2 agar (Cotty, 1989), and the number of *A. flavus* colonies was counted on modified rose bengal (MRB) agar (Cotty, 1994b). PDA and 5/2 agar were amended with streptomycin (0.05 g l<sup>-1</sup>) and chloramphenicol (0.05 g l<sup>-1</sup>). Fungal propagules m<sup>-3</sup> air were calculated. For comparisons between total fungi and quantity of *A. flavus* counts based on the 5/2 plates were used. Bacterial colony counts were made on unamended nutrient agar. After sub-culturing on 5/2 agar for 7 days at 31 C all *A. flavus* isolates were assigned to either the S or L strains on the basis of morphological criteria (Cotty, 1989). A total of 3,307 *A. flavus* isolates (c.25 per sample) were sub-cultured and characterized to strain. The proportion of *A. flavus* accounted for by the applied atoxigenic strain was estimated by vegetative compatibility group (VCG) analysis

(Cotty, 1994a) of the first 20 L isolates sampled every 4 weeks (720 isolates total). The growers provided cropping history of the four 40-acre fields immediately adjacent to each sampler.

The size range of particles sampled by the cyclone sampler was determined microscopically (Olympus X100 S Plan, dry objective, ocular micrometer) for seven separate samples. The sample was suspended in sterile distilled water and the first 100 particles observed during an 80  $\mu\text{m}$ -wide transect across the slide were measured. In a similar manner, conidia from 7-day old cultures of *A. flavus* (AF36) grown on 5/2 agar were measured to ensure conidia of this isolate fell within the size range of the particles being sampled by the Burkard cyclone sampler. The length and width of both fresh conidia suspended in sterile distilled water, and conidia dried in an oven at 50 C for 4 h were measured. Dried conidia were dusted directly onto slides.

Coverslips were used. Conidia were dried prior to measurement in an attempt to simulate dehydration of conidia in the Sonoran-desert of western Arizona.

Filter membranes were used to further characterize propagules of *A. flavus* sampled by the cyclone sampler. Two samples were suspended in 5 ml sterile distilled water and repeatedly filtered through polycarbonate screen membranes (Poretics Corporation, Livermore, California) with decreasing pore size (control - not filtered, 20, 12, 5, and 3  $\mu\text{m}$  pore size). These filters are perforated with precise pore size and have sharp cut-off retention characteristics (Ballew, 1997). The proportions of *A. flavus* propagules passing through each filter were determined by dilution plate technique.

**Filter impaction.** To compare efficiency of sample catches of the Burkard cyclone sampler with a membrane impaction technique, air pumps (12 v DC, P/N MTR1002, Gast Manufacturing Corporation, Benton Harbor, Michigan) fitted with membrane based field monitors for contamination analysis (pore size 0.8  $\mu\text{m}$ , Millipore Corporation, Bedford, Massachusetts) were operated at 16.5 L  $\text{min}^{-1}$  alongside the cyclone samplers for different periods. After sampling, the filter was removed and washed three times in 2 ml sterile distilled water. Three 0.1-ml aliquots of each washing were plated onto MRB. The membrane filter was also placed face up on the agar. The quantity of *A. flavus*  $\text{m}^{-3}$  air sampled was calculated. A cyclone sampler was

1 operated simultaneously with the impaction samplers and the cyclone samples were processed as  
2 previously described except the sample was suspended in 0.5 ml sterile distilled water and the  
3 entire volume was plated. Comparisons were made on three dates. For each date, three replicate  
4 samples were taken. Replicates consisted of one cyclone sample and three impaction samples  
5 taken simultaneously.

6 Rotorod samplers. The efficiency of rotorod samplers (Perkins, 1957) in collecting *A.*  
7 *flavus* propagules was also tested. Due to the inherent difficulty of identifying *A. flavus* based on  
8 spore morphology, modifications to allow culturing of the fungus were necessary. The usual  
9 "sticky" impaction surfaces of vaseline or silicone grease generally used for entrapping spores  
10 may be toxic to fungi and are not soluble in water and thus are not readily amenable to dilution  
11 plate technique. A water-soluble material was developed. A mixture of carboxy-methylene  
12 cellulose (CMC, 14 g), Glycerol (20 ml) and water (40 ml) formed a stiff, tacky grease that  
13 spread evenly on 0.1 cm dia. quartz glass collecting rods (Friedrich and Dimmock Glassworks,  
14 Millville, New Jersey). The coating was applied by sliding the rod between index finger and  
15 thumb. On plastic or metal the coating coalesced, preventing even distribution on the sampling  
16 face. Sampling characteristics were compared with silicone grease by operating the rotorods in  
17 flow hoods while gently tapping an upturned plate to dislodge conidia. One rod was coated with  
18 the CMC coating, the other with silicone grease. The rods (2.5 cm long) were removed after  
19 operation and the number of conidia sampled counted on each rod. The glass rods (16 cm apart)  
20 were placed in 1 mm holes drilled in the ends of the plastic tube of a ball point pen. Locking  
21 nuts were glued on the end and used to hold the rods in place. Attached to the center of the  
22 plastic tube was the bevel with lock nut to attach the tube to the motor spindle. The apparatus  
23 rotated at 3000 rpm.

24 For field sampling the rotorod motors (12 v DC, Model Number 2M197, Dayton Electric  
25 Manufacturing Company, Niles, Illinois) were supported within 10 cm (internal diameter)  
26 polyvinylchloride pipe joints (Fig. 3). Three motors were placed on each pipe by using a four-

way pipe split with 50 cm sections of tube placed in the horizontal outlets and motors placed in the open ends of the pipes. Thus each experiment had three replications. The base of the piping was buried in the ground. Sampling period was investigated by operating the motors for 2 and 4-h periods. This was repeated twice. In addition samples were taken at different locations and times.

**Weather data.** Weather data was obtained from the Roll station of the Arizona Meteorological Network (AZMET) at <http://ag.arizona.edu/azmet/24html> (files 2498ew.txt and 2499ew.txt). Details of the equipment and measurement protocols used at the Roll station may be found at this site. The mean weekly data for temperature (maximum, minimum, and mean); rainfall and humidity were used to describe the weather conditions for the duration of the sampling period from May 1997 to March 1999.

1        Soil and surface samples. In late June and October of 1997 samples of soil, leaves, leaf  
2        litter, and boll material were taken from fields adjacent to the cyclone sampler in the treated area.  
3        Five replicate samples were taken at random points within 50 m of the sampler. Soil samples  
4        comprised the surface 2-3 mm of soil scraped from a 2 m length of row. The lower and oldest  
5        leaves (100 g) were collected in June and in October chemically defoliated (as per normal  
6        commercial practice) leaves (100 g) were collected from the ground. Boll samples (20  
7        bolls/sample) were taken at random locations within 50 m of the sampler. In June bolls were  
8        immature and unopened. In October bolls were fully mature and open. Samples were taken  
9        from the middle to bottom of the canopy.

10       In June and October of 1998, soil samples were taken from the four fields surrounding each  
11       cyclone sampler. Samples were taken along a diagonal across each field starting at that site's  
12       sampler at 25, 50, 150, 250 and 400 m, as described above.

13       Soil samples were mixed thoroughly and a subsample (5-20 g) was suspended in 50 ml  
14       sterile distilled water, agitated vigorously for 10 min, and subjected to dilution plate technique on  
15       MRB agar. Leaf material (5-8 g in 200 ml), leaf litter (5-17 g in 500 ml) and bolls/locules (10-  
16       25 g in 500 ml) were processed similarly. CFUs of *A. flavus* were counted and CFUs g<sup>-1</sup> of  
17       material calculated. Strain (S/L) composition and the proportion of the L strain in the applied  
18       VCG was estimated as for the cyclone samples.

19       **Data analysis.** Data was analyzed using Statistica V3.0 and SAS V8.0. Comparisons  
20       between microbe counts at treated and untreated sites were with t-tests. These included  
21       quantitative comparisons of total fungi, *A. flavus*, *A. niger*, bacteria, S strain, and the applied  
22       atoxigenic VCG. Correlation analyses were used to relate total fungal propagules with those of  
23       *A. flavus*, and to investigate the relationship between rotorod sampling methods (silicon grease  
24       vs CMC). Analysis of variance was used to test all multiple comparisons (catches of *A. flavus* by

filter impaction vs cyclone sampler, catches of *A. flavus* by rotorods, and propagule counts and characteristics from soil, bolls and leaves). Tukey's HSD test was used to separate the means. Standard deviations were calculated for the size ranges sampled by the Burkard cyclone samplers, and the numbers of CFUs obtained after filtering the cyclone sampler catches.

## RESULTS

**Cyclone samplers.** The MRB agar tended to grow more *A. flavus* colonies than either the PDA or 5/2 media (Table 1). However, except for the PDA vs MRB at the untreated site, t-tests showed no significant differences, probably on account of the large variances associated with each of these samples. Counts of total fungal propagules were made on 5/2 (a mean of 139.5 and 120.3 propagules  $m^{-3}$  at the treated and untreated sites, respectively) and PDA (a mean of 119.8 and 89.6 propagules  $m^{-3}$  at the treated and untreated sites, respectively). As there was no significant difference between the counts on the two media (t-test,  $P=0.05$ ), only those for 5/2 are presented.

The weight of material collected by the cyclone samplers varied from 0.001 g to 1.0018 g per sampling period (Fig. 4A). However, the quantity exceeded 0.2 g on only two occasions. Numbers and characteristics of propagules sampled from the two sites were similar (Fig. 4B). During 1997 (May-December) the total fungal propagules collected at the non-treated site ranged from 17 to 667  $m^{-3}$ , and at the treated field from 9 to 1277  $m^{-3}$ . During 1998-99 (January 1998 – March 1999) total fungal propagules ranged from 2 to 652 and 2 to 412 propagules  $m^{-3}$ , respectively at the two sites. Over the same time periods counts of *A. flavus* in 1997 ranged from <1 to 406 and <1 to 416  $m^{-3}$  at the treated and untreated sites respectively, and in 1998-9 the numbers ranged from <1-361 and <1-117, at the two sites respectively. On 5/2 agar *A. flavus* comprised <1 to 46% of the total cultured fungi at the untreated site and <1 to 51% at the treated site in 1997 (Fig. 4C), and <1 to 41% of the total cultured fungi at the non-treated site and <1 to 44% at the treated site in 1998. Greatest numbers of both total fungal propagules and *A. flavus* propagules occurred between JD 177 in 1997 to JD 43 in 1998 and JD 191 in 1998 to JD 8 in

1 1999. In both years the peaks coincided with JD 251-32. Very low numbers of all propagule  
2 types were sampled between mid January and May (sample weights at these times also tended to  
3 be lowest).

4 There were no significant differences ( $t=ns$ ,  $P = 0.05$ ) between the two sites in either mean  
5 number of total propagules  $m^{-3}$  nor propagules of *A. flavus*  $m^{-3}$ . A mean of 139.5 and 120.3 total  
6 fungal propagules  $m^{-3}$  were sampled at the treated and untreated sites respectively ( $t=ns$ ,  
7  $P=0.05$ ). *Aspergillus flavus* propagule counts were 28.6 and 29.7  $m^{-3}$  at the treated and untreated  
8 sites, respectively ( $t=ns$ ,  $P=0.05$ ). Differences between catches at the two sites in the quantity of  
9 *A. flavus* were not detected following application of the atoxigenic VCG. The quantity of *A.*  
10 *flavus* collected by the treated site sampler in 1997 (JD194-343) differed from that collected in  
11 1998 (JD 194-341) (100.2 versus 19.3 propagules  $m^{-3}$ ,  $t=2.4780$ ,  $df=17$ ,  $P=0.01$ ). All four fields  
12 surrounding the sampler at the treated site were rotated from cotton in 1997 to wheat in  
13 December 1997 and then to lettuce during the 1998 period in question. At the untreated site  
14 samplings did not differ between these two periods (53.3 propagules  $m^{-3}$  and 54.2 propagules  $m^{-3}$   
15  $^3$ ,  $t=ns$ ,  $P=0.5$ ). These relationships were similar for total numbers of propagules collected  
16 (314.3 and 103.1 propagules  $m^{-3}$  at the treated site in 1997 and 1998, respectively,  $t=2.5603$ ,  
17  $df=17$ ,  $P=0.01$ , and 194.8 and 99.8 propagules  $m^{-3}$  at the untreated site,  $t=ns$ ,  $P=0.05$ ,  
18 respectively).

(cotton, wheat, lettuce?)

19 Correlation analysis showed a strong correlation between total number of fungi sampled and  
20 the number of *A. flavus* propagules at the treated site ( $r=0.9515$ , 152  $df$ ,  $P<0.001$ ). At the  
21 untreated site there was also a positive correlation ( $r=0.2294$ , 152  $df$ ,  $P<0.05$ ), although it was  
22 less pronounced. Thus conditions favoring production and dispersal of *A. flavus* were conducive  
23 for other fungi which increased in number at similar times.

24 *Aspergillus niger* propagules were also collected throughout the sampling period. Counts of  
25 *A. niger* were made on 5/2 (a mean of 33.9 and 23.9 propagules  $m^{-3}$  at the treated and untreated  
26 sites, respectively) and PDA (a mean of 26.2 and 21.1 propagules  $m^{-3}$  at the treated and untreated  
27 sites, respectively), as the MRB inhibited growth of *A. niger*. As there was no significant

1 difference between the counts on the two media ( $t=ns$ ,  $P=0.05$ ), only those for 5/2 are given. The  
2 counts followed trends similar to *A. flavus* with peaks of catches occurring during summer and  
3 autumn and with very low numbers during the winter. Propagule numbers of *A. niger* ranged  
4 from 0–273 propagules  $m^{-3}$ . The relative proportions of *A. niger* and *A. flavus* are shown in Fig.  
5 4D. Over the sampling period there were no significant differences ( $t=ns$ ,  $P = 0.05$ ) between the  
6 two sites in mean number of *A. niger* propagules  $m^{-3}$ . However, the quantity of *A. niger*  
7 collected by the treated site sampler in 1997 (JD194-343) differed from that collected in 1998  
8 (JD 194-341) (133.2 versus 15.7 propagules  $m^{-3}$ ,  $t=4.2813$ ,  $df=12$ ,  $P=0.001$ ). The quantity of *A.*  
9 *niger* collected by the untreated site sampler in 1997 (JD194-343) also differed from that  
10 collected in 1998 (JD 194-341) (62.7 versus 18.7 propagules  $m^{-3}$ ,  $t=2.5962$ ,  $df=17$ ,  $P=0.009$ ).

11 Large quantities of bacteria were sampled throughout the two-year study (Fig 4E). The  
12 number of bacterial propagules exceeded fungal propagules at all times ranging between ca.  
13 5000 and over ca. 50000 propagules  $m^{-3}$ . The counts appeared to be greater in the summer  
14 periods, with lower numbers sampled during the winter. There were no significant differences  
15 between the two sites in mean number of bacteria  $m^{-3}$  (2207 and 2364 propagules  $m^{-3}$  sampled at  
16 the treated and untreated sites respectively ( $ns$ ,  $t$ -test,  $P=0.05$ ). The quantity of bacteria  
17 collected by the treated site sampler in 1997 (JD194-343) differed from that collected in 1998  
18 (JD 194-341) (1882 vs 768 propagules  $m^{-3}$ ,  $t=2.2433$ ,  $df=19$ ,  $P=0.018$ ). However, the quantity of  
19 bacteria collected by the untreated site sampler in 1997 (JD194-343) was not significantly  
20 different to that sampled in 1998 (JD 194-341) (4195 versus 2335 propagules  $m^{-3}$ ,  $t=ns$ ,  $P=0.05$ ).  
21 Other fungi (including *Fusarium* spp., *Alternaria* sp. and *Cladosporium* sp.) were sampled but  
22 counts and identifications were not made on these species which were generally individually less  
23 numerous than either *A. flavus* or *A. niger*.

24 Both S and L strain isolates of *A. flavus* were detected at both the treated and untreated sites  
25 (Fig. 5A). Overall, there was a greater proportion of the L strain at the treated site (80.2%)  
26 compared to the untreated site (68.5%,  $t=2.5390$ , 135  $df$ ,  $P=0.006$ ). There was distinct  
27 seasonality in the incidences of the S and L strains with the L strain most abundant from

1 September through December. At the treated site 81.3% and 74.3% of the *A. flavus* was L strain  
2 between JD 147 and 360 in 1997 and 1998, respectively ( $t=ns$ ,  $P=0.05$ ), and at the untreated site  
3 over the same period 68.9 and 63.1% was L strain, respectively ( $t=ns$ ,  $P=0.05$ ). Greatest  
4 quantities of S occurred between May and August both years (35.7-96.0% S (1997) and 28.0-  
5 100% S (1998) at the untreated site and 4.5-100% S (1997) and 16.7-84.0% S (1998) at the  
6 treated site). The proportion of S at the treated site between May and August (JD 147-243) in  
7 1997 (30.5%) differed from that present between September and December (JD 148-365)  
8 (3.9%), ( $t=4.16$ , 14 df,  $P<0.001$ ). Similar differences occurred in 1998 (38.6% versus 15.5%,  
9  $t=2.96$ , 19 df,  $P=0.004$ ) at the treated site and in both years at the untreated site (54.7% versus  
10 5.41% in 1997 ( $t=6.2410$ , 13 df,  $P<0.001$ ) and 56.5% versus 17.2%, in 1998 ( $t=4.88$ , 17 df,  
11  $P<0.001$ )). The applied atoxigenic VCG belongs to the L strain, and accounted for 0-47% of  
12 the total L strain sampled at the non-treated site, and 5-75% at the treated field (Fig. 5B). The  
13 applied atoxigenic VCG was more prevalent in the air at the treated site than at the untreated site  
14 ( $t=-3.2041$ , 32 df,  $P<0.01$ ).

15 **Cropping history.** During the sampling period various crops were grown in the fields  
16 adjacent to the two cyclone samplers (Table 2). At the treated site the four fields surrounding the  
17 sampler were rotated from cotton to wheat and then to lettuce and back to wheat. Cotton was  
18 only present at the treated site during 1997. All four fields surrounding the sampler at the  
19 untreated site were rotated from barley to cabbage and then to cotton followed by wheat. The  
20 two sites only had the same crop (wheat) from December 1998 through March 1999. During the  
21 1998 sampling period the non-treated site was planted to cotton, although the early cotton season  
22 was very cool resulting in poor crop development and one of the lowest yielding Arizona crops  
23 in history.

24 **Weather data.** Data from the AZMET station at Roll (Fig. 6) indicate that in both 1997 and  
25 1998 maximum daytime temperature was greatest during the summer between JD 100-300 (30-  
26 45 C). The minimum temperature did not fall below 10 C and was generally above 15 C.  
27 Rainfall was scattered scantily throughout the sampling period with showers rarely exceeding 1

cm. Maximum relative humidity ranged from 50-100% and tended to be higher during the winter. All crops in Western Arizona are irrigated and irrigation may have exerted greater influence than rain on the microbial communities sampled.

**Particle size.** Sizes of particles collected by the Burkard cyclone sampler during this study and measured microscopically are summarized in Fig. 7. Particles from 1  $\mu\text{m}$  to 2.5  $\mu\text{m}$  occurred at the greatest frequency. Although observed particle sizes ranged from  $<1 \mu\text{m}$  to  $>30 \mu\text{m}$ . A few larger particles were occasionally observed, although not in the samples examined microscopically. These larger particles were generally whole insects or insect parts that were entrapped in the sampler. Microscopic examination revealed both organic (spores, plant and animal debris) and inorganic particles (dust, sand). Most propagules of *A. flavus* were within the range expected for conidia (Fig. 8). Few propagules passed through pores of 5  $\mu\text{m}$  or less, but the quantity that passed through 12  $\mu\text{m}$  pores was not significantly different from the control.

**Size of *Aspergillus flavus* spores.** Seven-day-old conidia of the applied atoxigenic VCG grown at 31 C on 5/2 agar were spherical and ranged from 3.2  $\mu\text{m}$  to 5.2  $\mu\text{m}$  in diameter. Oven dried conidia were ovoid and crumpled and ranged from 2.4  $\mu\text{m}$  to 5.6  $\mu\text{m}$  x 1.76  $\mu\text{m}$  to 3.6  $\mu\text{m}$ .

**Cyclone versus impaction sampler.** The number of propagules sampled by impaction onto filters versus by the cyclone sampler is shown in Table 4. Analysis of variance did not indicate differences between the quantity of conidia sampled by the two methods for any of the sampling dates. However, there was great variability among samplings possibly related to the short duration of the sampling period (2 hr).

**Rotorod samplers.** In lab studies the CMC-glycerol coating was apparently as effective as the silicone grease at entrapping conidia of *A. flavus* (Fig. 9A and B). An analysis of variance showed no difference in the number of conidia sampled using the two coatings, and there was a positive correlation between values for the two coatings for both the quantity of conidia sampled (0.7322,  $P=0.01$ ) and the frequency of encounters with conidia (0.9501,  $P=0.001$ ). Cluster sizes of conidia sampled were comparable under both conditions (Fig. 9C). However, viability of conidia sampled in the lab was not quantified. In the field, a time period of exposure test showed

1 that overloading of the rotorods probably occurred after 2 h sampling (Table 5). In both  
2 sampling period tests the 4 h period sampled fewer propagules  $\text{m}^{-3}$  than the sum of the  
3 consecutive 2 h sampling periods. Although propagules of various fungi (*Alternaria*,  
4 *Cladosporium* and *Aspergillus* spp) were consistently sampled, there were few encounters with  
5 viable propagules of *A. flavus* at these times. Only *A. niger* was sampled consistently, however,  
6 at different sampling times the quantity of viable propagules sampled varied substantially from  
7  $<1\text{-}56$  propagules  $\text{m}^{-3}$ .

8 *Soil, leaf and debris population counts.* In 1997 quantities of *A. flavus* on cotton leaves, in  
9 soil, and on bolls were compared at the treated site in June and October (Table 6). Analysis of  
10 variance indicated differences among substrates in both number of propagules ( $F=4.35$ ,  $P=0.006$ )  
11 and the prevalence of S-strain propagules ( $F=676$ ,  $P<0.0001$ ). Leaf-litter had the most *A. flavus*  
12 ( $2.7 \times 10^5$  propagules  $\text{g}^{-3}$ ), and the greatest proportion of S strain (17.3%). Bolls also had large  
13 quantities of *A. flavus* propagules ( $6.9 \times 10^3\text{-}1.5 \times 10^4$  propagules  $\text{g}^{-3}$ ). In 1998 only soils at the  
14 treated and untreated sites were compared (Table 7). The number of soil propagules and the  
15 proportions of both the S strain and the applied VCG differed significantly ( $P<0.001$ , ANOVA)  
16 between sites. In June there was a mean of 229 propagules  $\text{g}^{-1}$  soil at the treated site, but by  
17 October there were only 58 propagules  $\text{g}^{-1}$  of soil. However, at the untreated site propagules per  
18 gram shifted from 418 in June to over  $1.6 \times 10^4$  in October (Table 7). In October the treated site  
19 was in lettuce and the untreated site was in cotton (Table 2). In 1997, the S strain was not  
20 detected in soil from the treated site and the S strain composed just over 1% of isolates recovered  
21 from any substrate at that site. The S strain was more common at the treated site in 1998 with  
22 14% of isolates belonging to the S strain in June compared with 67% at the untreated site. At the  
23 treated site in 1997, the applied VCG accounted for 92.7% and 81.3% of the L strain isolates in  
24 June and October, respectively. In 1998 the proportion was 81% in June and 66% in October.  
25 At the untreated site in 1998 the applied VCG accounted for 43% and 33% of the L strain in June  
26 and October, respectively (Table 7).  
27

## DISCUSSION

This study describes the dynamics of air-borne microbial propagules in cultivated desert regions of southwestern Arizona. Emphasis is placed on *A. flavus* and influences on the air flora of applications of an atoxigenic *A. flavus* VCG currently used for aflatoxin management in Arizona. Various aspects of the dispersal phase of the epidemiology of *A. flavus* in several crop systems and environments have been investigated (Morrow et al., 1964; Holtmeyer and Wallin, 1980 and 1981; Olanya et al., 1989), and some prior information on aerial dispersal of *A. flavus* in Arizona is available (Lee et al., 1986). However, this is the first time the year-round characteristics of air-borne propagules of *A. flavus* have been described in an agricultural setting during seasons when epidemics of aflatoxin contamination of a crop occurred. In both seasons cottonseed produced throughout this local region experienced aflatoxin contamination at economically damaging levels (Cotty, unpublished data). The severity and distribution of aflatoxin contamination within this area in 1995 and 1996 has been detailed (Bock and Cotty, 1999). The current study shows *A. flavus* is a major component of the air around cotton fields of southwestern Arizona during the summer and autumn periods and it is a common component of air around rotation crops as well. Various other fungi (*Fusarium* spp., *Alternaria* spp. and other *Aspergillus* spp.), and bacteria are also common constituents of the air surrounding agricultural fields in this area.

*Aspergillus flavus* was counted on a selective medium (MRB) to ensure detection of the maximum number of *A. flavus* propagules. The other fungi (including *A. niger*) did not have this advantage and thus they were undoubtedly undercounted. Indeed, fewer *A. flavus* propagules were detected on either 5/2 or PDA than on MRB. This agrees with a previous report on *A. flavus* isolation using MRB (Cotty, 1994b). There are probably large numbers of fungi that were either uncultivable on the media we employed or were competitively excluded from the culture plates by more aggressive species. We incubated our isolation media at a relatively high temperature (31 C) that may have been inhibitory to many fungal taxa, particularly those that thrive during the winter when our counts of fungi were low.

1 Lee et al. (1986) previously found large quantities of airborne *A. flavus* propagules associated  
2 with cotton production on individual sampling dates in July to September. However, the Lee  
3 study had highly variable results, perhaps associated with the brief sampling periods allowed by  
4 the Anderson sampler used in that study. In the current study, peaks in total fungal propagules,  
5 including *A. flavus* and *A. niger*, occurred between JD 251–321 (September to November).  
6 “Shoulder” periods with fewer propagules occurred before and after these dates. The peak  
7 periods for catches of all microorganisms were associated with extensive, valley-wide cotton  
8 harvest activity and subsequent cultivation associated with stalk-shredding and plow-down  
9 (mandated for insect control) and preparation of soils for winter produce crops. Chemical  
10 defoliation, harvest, and plow-down create a huge source of organic debris, which support fungal  
11 growth. In Arizona, during these periods, temperature maxima frequently exceed 30 C and  
12 temperatures remain for most of the day in a range favorable to growth of *A. flavus* (Ashworth et  
13 al., 1969; Ayerst, 1969; Marsh et al., 1973; Diener et al., 1987) and similarly adapted fungi.  
14 Aflatoxin contamination of cottonseed is particularly common in high temperature regions  
15 (Marsh et al., 1973). Sufficient moisture for fungal reproduction, infection, and aflatoxin  
16 contamination may be readily available from late season irrigation, rainfall and dew (Bock and  
17 Cotty, 1999; Russell et al., 1976).

18 Cotton is an indeterminate plant and in Arizona where long-season cotton is common, the  
19 earliest bolls may mature and open months before the crop is harvested. Large numbers of *A.*  
20 *flavus* propagules were caught during the periods of cotton boll formation, maturation, and  
21 harvest. Prevalence of inoculum does not appear to limit cottonseed infection during these  
22 periods. Thus, in Arizona, predisposition of the crop is apparently more important than presence  
23 of inoculum in dictating which bolls become infected. In the first phase of contamination  
24 (during boll development; Cotty, 1997) bolls are typically predisposed by insect damage (Cotty  
25 and Lee, 1989). During the second phase (after boll maturation) predisposition occurs when the  
26 crop is exposed to warm moist conditions (Ashworth et al., 1969, 1969 and 1971; Diener et al.,  
27 1987; Cotty, 1991). The large quantities of conidia in the air observed in the current study

1 provide constant exposure of open bolls to increasing loads of *A. flavus* propagules. As crops are  
2 held in the field later in the season, there are increasing opportunities for crop predisposition to  
3 infection by exposure to humidity events during high temperature periods. This may explain the  
4 greater aflatoxin content observed in crops harvested later in the season (Bock and Cotty, 1999).  
5 Indeed, the large quantities of *A. flavus* observed later in the season support the view that later  
6 harvests suffer increased contamination – not just from early infections producing more  
7 aflatoxin, but also from new infections (Bock and Cotty, 1999).

8 The applied atoxigenic VCG belongs to the L strain and is endemic to the test area and  
9 ranged from <1% to 4% of *A. flavus* in the soils of this area prior to application in 1996 (Cotty,  
10 unpublished data). The treated site appeared to have a significantly greater proportion of the L  
11 strain which may be due to competitive exclusion of the S strain by the applied VCG (Cotty,  
12 1994a). We do not have data for previous seasons, but the S strain was more prevalent at the  
13 non-treated site than at the treated site, and the proportion of the L strain did not appear to  
14 change significantly between 1997 and 1998 suggesting that the effect of treatment did not  
15 disappear after a single season. This agrees with previous observations (Cotty, 1999). The  
16 relatively high incidence of the applied VCG at the untreated site (up to 47% of the L strain at  
17 the untreated site was the applied VCG) probably resulted from dispersal from the site treated in  
18 1996 and 1997 (the distance between the edge of the treated area and the sampler in the non-  
19 treated site was about 1 km). Quantities of *A. flavus* propagules sampled at both the treated and  
20 untreated sites were similar throughout the 1997 season and thus, application of the atoxigenic  
21 VCG apparently altered the composition of *A. flavus* in the air, without altering the total quantity  
22 of *A. flavus*. Indeed, air spora from other surrounding areas may well influence the spectrum of  
23 spores sampled, and various factors including wind speed and direction and farming activity will  
24 affect the propagule type and quantity sampled at a particular time. One advantage of the  
25 cyclone sampler is that continuous sampling over 1-week periods is possible, thereby  
26 overcoming the effects of transient changes of wind direction or speed which could influence  
27 results from samplers operated for short periods.

1       The most likely source of the relatively high incidence of the applied VCG at the untreated  
2 site are the atoxigenic VCG applications. Previous studies (Olanya et al., 1997) have shown  
3 linear dispersal gradients of air-borne inoculum of *A. flavus* from waste corn piles in Iowa, with  
4 propagules being dispersed at least 14 m. In our study the atoxigenic VCG was applied to a 16  
5 ha block in 1996 and a 65 ha block in 1997, which constitutes a large source of inoculum for  
6 aerial dispersal over a period of two years. It is also possible that insects play a role in dispersing  
7 the fungus (Olanya et al., 1997). The fact that *A. flavus* is aeri ally dispersed and can probably  
8 travel at least several hundred meters lends support to the use of area-wide applications to  
9 establish the atoxigenic VCG and minimize ingress of aflatoxin producing strains from other  
10 habitats. This approach would provide the additional benefit to farming communities of  
11 reducing contamination of all crops grown in a region. It would also reduce any health risk  
12 associated with aflatoxin exposure via respiration (Oyelami et al, 1997; Autrup, et al, 1993) of  
13 either conidia containing aflatoxin (Wicklow and Shotwell, 1983), or crop associated dust (Lee  
14 et al., 1983; Salim et al, 1998).

15       Long range movement of *A. flavus* may also be suggested by failure of the air catches to  
16 closely mirror the soil composition at either the treated or not-treated sites. High incidences of  
17 the S strain were caught at the treated site in 1998 (JD 160 to JD 253) even though soil analyses  
18 of the four fields surrounding the sampler indicated a low incidence of the S strain. Either S  
19 strain propagules were dispersed to the sampler from beyond the treated area (1 to 1.5 km) or  
20 there was preferential dispersal of the S strain from soils during certain periods. Seasonal  
21 increases in S strain incidence in soils of western Arizona in July/August have previously been  
22 described (Orum et al. 1997). However, the very low levels of S strain isolates in the soils of  
23 treated fields suggest compositional differences between cyclone and soil samples reflect  
24 movement of both the S strain into the treated area and the applied atoxigenic VCG into the non-  
25 treated area. There are diverse crops in this region and propagules may have originated from  
26 agricultural fields undergoing soil preparation or harvest. However, it is also possible that S  
27 strain propagules originated from native desert areas which greatly outweigh cultivated regions

1 in the test area and which have significant *A. flavus* communities (Boyd and Cotty, 1998).

2 We found the S strain was prevalent in the air from May through December (peaking from  
3 July to September) which spans the period of boll development and maturation and may be  
4 important to the epidemiology of aflatoxin contamination of cottonseed. The S strain produces  
5 comparatively greater amounts of aflatoxin than the L strain (Cotty, 1989), and readily infects  
6 cotton bolls (Garber and Cotty, 1997). Although the origin of the large proportion of S strain  
7 observed in summer and early fall remains unclear, the plume begins before cotton boll  
8 maturation and declines during cotton crop harvest. It is thus unlikely, at least initially, to  
9 originate from within the current crop. The S strain produces large quantities of sclerotia (Cotty,  
10 1989, Garber and Cotty, 1997) and the plume may reflect release of conidia from sclerotia  
11 formed on trash from prior crops. Alternatively the S strain propagules may come from soil  
12 disturbance during either harvest or incorporation of crop debris from alternative crops (i.e.  
13 wheat).

14 The filtration studies suggested that *A. flavus* propagules collected by the cyclone sampler  
15 were, for the most part, in the range of conidia (<12  $\mu$ m diameter), rather than larger sclerotia or  
16 colonized organic debris. However, air transport of sclerotia from colonized locules (Garber and  
17 Cotty, 1999) to the soil must occur and it seems likely some movement of sclerotia between  
18 fields occurs. Sclerotia of the S strain are relatively small (S originally designated small sclerotia  
19 <400  $\mu$ m) and dust storms are common in this region. Large quantities of soil and associated  
20 organic matter can be driven over vast areas by storms and sclerotia of the S strain would be  
21 dispersed.

22 Both the crop and season greatly influence the magnitude of *A. flavus* soil communities  
23 (Orum et al., 1997). In 1997 cotton was produced in a warm season typical of the area. However,  
24 spring and early summer of 1998 were uncharacteristically cool in southwest Arizona. This  
25 resulted in both the worse cotton crop in memory and reduced conditions favorable to *A. flavus*  
26 growth. Significantly less *A. flavus* was sampled at the treated site in 1998. The four-fold  
27 reduction in the quantity of *A. flavus* in the soil observed at the treated site between June (wheat

1 planted) and October (lettuce planted) was probably due to both the cooler year and the change  
2 in cropping. Cotton production in Arizona favors development of high densities of *A. flavus*  
3 propagules (Orum et al., 1997) and at the non-treated site *A. flavus* propagules in the soil  
4 increased under cotton production forty-fold between June and October 1998. However, the  
5 quantity of propagules captured by the cyclone sampler at the non-treated site did not differ  
6 between 1997 (when cabbage was grown) and 1998. Failure to see an increase in air-borne  
7 propagules associated with cotton production is probably attributable to both the cooler year and  
8 the reduced amount of cotton immediately adjacent to the non-treated site in 1998 versus 1997.  
9 Thus, cotton production outside of the site may have contributed captured propagules to a greater  
10 extent in 1997 than in 1998.

11 To the best of our knowledge this is the first report of a Burkard cyclone sampler being used  
12 to sample fungal propagules for studying a plant pathogen. The equipment has many useful  
13 aspects that make it practical in a study of plant pathogens, particularly in dry environments.  
14 Cyclone samplers can be operated continuously for long periods of time (this equipment ran  
15 continuously from May 1997 through March 1999 using solar powered batteries). Operation  
16 over long periods prevents wide transient fluctuations in propagule numbers from having a major  
17 impact on overall counts. Cyclone samplers collect a dry dust that allows ready quantification of  
18 viable propagules through culture on appropriate media. This is particularly useful where size or  
19 spore characteristics make identification difficult. The size of conidia (in our study 3.2-5.2  $\mu$ m in  
20 diameter) and similarity of spores of *A. flavus* to other *Aspergilli* make it difficult to assess them  
21 visually, and counting morphologically distinct colony forming units is a more certain approach.  
22 The size ranges of particles sampled in our study also suggest that cyclone samplers would be  
23 suitable for sampling many other fungal spores. Numbers of cultivable *A. flavus* propagules  
24 caught by the cyclone samplers were comparable to those caught with the filter impaction  
25 samplers previously shown to be the most efficient of four air samplers tested for detection of  
26 air-borne *A. flavus* spores (Silas, et al, 1986). However, we were unable to sample *A. flavus*  
27 propagules in the field using rotorods. Rotorods coated with a carboxy-methyl cellulose based

grease did sample other fungi including *A. niger*. It may be that conidia of *A. flavus*, under field conditions, were rendered non-cultivable by impact on the rotorods. Nonetheless, the rotorods were successful at quantifiably capturing culturable *A. niger* at propagule concentrations similar to those captured by the cyclone sampler.

The formulation currently being used to treat areas of the Mohawk Valley is sterile wheat seed colonized with an atoxigenic *A. flavus* (Bock and Cotty, 1999). Timing of application is an important criterion to maximize the production of conidia while minimizing the risk of product loss to predation or burial. Timing of conidia release from the product is crucial to optimize competitive exclusion of aflatoxin producing strains of *A. flavus* (Cotty and Bayman, 1993).

Data presented here suggest *A. flavus* becomes a noticeable component of the air spora by early May. On the basis of this observation, earlier treatments are likely to be more effective, providing temperature and moisture do not limit fungal metabolism and production of conidia. Treatments as early as the first week of May should be considered. At this time other strains of the fungus shall not have had chance to reproduce or colonize substrates and the applied atoxigenic VCG should have increased competitive advantage.

Data presented here suggest that application of an atoxigenic VCG of *A. flavus* at the currently used rate (10 kg colonized wheat seed ha<sup>-1</sup>) does not significantly increase the overall quantity of *A. flavus* in the air at treated sites as compared to untreated areas. In areas of Arizona where aflatoxin contamination of cottonseed is a perennial problem, *A. flavus* is a major component of the airspora and appears to be dispersed over distances of at least several hundred meters. Early, area-wide application of an atoxigenic VCG would thus make sense for effective aflatoxin control in these intensive agricultural areas in the desert environments of Arizona. Indeed, early application may be necessary to optimize efficacy of applications.

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- 5 and sclerotia of *Aspergillus flavus* and *Aspergillus parasiticus*. *Can. J. Microbiol.* 29:1-5.

1 TABLE 1. Colony forming units (CFU) of *Aspergillus flavus* cultured on different isolation  
2 media from samples collected by a cyclone sampler

Field treatment	Media <sup>a</sup>	Mean (CFU/m <sup>-3</sup> ) <sup>b</sup>	T-test comparisons	
			Media	t-value, df, P-value
Treated	PDA	17.5	PDA vs 5/2	-0.88, 99, 0.19
	5/2	24.1	5/2 vs MRB	0.73, 109, 0.23
	MRB	32.5	MRB vs PDA	1.45, 80, 0.08
Untreated	PDA	15.7	PDA vs 5/2	-0.18, 119, 0.43
	5/2	16.7	5/2 vs MRB	1.53, 94, 0.06
	MRB	33.0	MRB vs PDA	1.70, 84, 0.05

3

4 <sup>a</sup>media as described in the Materials and Methods.

5 <sup>b</sup>mean CFU/m<sup>-3</sup> from weekly enumeration of propagule counts in air sampled May 1997 through

6 March 1999 as described in the Materials and Methods.

1 TABLE 2. Cropping history<sup>a</sup> of the fields adjacent to the Burkard cyclone sampler at the treated and untreated sites in the Mohawk  
2 Valley, Arizona

	1997										1998										1999			
Field	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	
Treated	ct	ct	ct	ct	ct	ct	fw	wt	wt	wt	wt	wt	wt	wt	fw	lc	lc	lc	lc	wt	wt	wt	wt	
Not-treated	ba	fw	fw	ca	ca	ca	ca	ca	ca	fw	ct	ct	ct	ct	ct	ct	ct	ct	fw	wt	wt	wt	wt	

3  
4 <sup>a</sup>ct = cotton, ca = cabbage, lc=lettuce, fw=fallow, soil preparation for next crop, wh=wheat, ba=barley.  
5  
6

1 TABLE 3. The dimensions of fresh and dried conidia of *Aspergillus flavus*

Treatment	Length (μm)			Width (μm)		
	Mean	Max	Min	Mean	Max	Min
	(st dev)			(st dev)		
Fresh	4.22 (0.46)	5.2	3.2	-- <sup>a</sup>	--	--
Dried <sup>b</sup>	3.33 (0.42)	5.6	2.4	2.92 (0.42)	3.6	1.76

2

3 <sup>a</sup>fresh conidia are spherical.

4 <sup>b</sup>measured after drying in an oven at 50 C.

1 TABLE 4. The quantity of *Aspergillus flavus* propagules in the air sampled either by  
2 impaction onto membranes or by a Burkard cyclone sampler in the Mohawk Valley, Arizona,  
3 1998

Test	Date	Propagules sampled m <sup>-3</sup>	
		Cyclone	Impaction
1	June 17	51 <sup>a</sup>	29
2	November 18	103	583
3	November 19	389	98
Three Test Mean		181	236

4  
5  
6 <sup>a</sup>for each date cyclone values do not differ significantly from impaction values by analysis of  
7 variance. Values are means of three replicates. Cyclone replicates are single measurements;  
8 each impaction replicate is the mean of three measurements taken simultaneously.  
9

1 TABLE 5. The numbers of fungal propagules (total fungi and *Aspergillus niger*) sampled by  
2 the rotorod samplers in the Mohawk Valley, Arizona, 1998

Date	Duration of sampling (h)	Rotorod sampler no.	Total fungi	<i>A. niger</i>
			Propagules/m <sup>3</sup> (stdev) <sup>b</sup>	Propagules/m <sup>3</sup> (stdev) <sup>b</sup>
18/11/98	2	1	11.8 (1.5)	- <sup>a</sup>
	2	2	46.0 (26.5)	-
	4	3	28.0 (7.7)	-
18/11/98	2	1	3.1 (1.5)	-
	2	2	3.9 (0.4)	-
	4	3	2.7 (1.9)	-
18/11/98	2	1	8.5 (2.4)	-
	2	2	9.8 (3.0)	-
19/11/98	2	1	11.8 (6.0)	3.4 (3.0)
	2	2	9.1 (0.9)	2.5 (1.9)
19/11/98	2	1	24.3 (0.7)	15.0 (5.5)
	2	2	10.1 (5.2)	5.7 (4.7)
19/11/98	2	1	56.2 (25.2)	4.2 (2.4)
	2	2	50.3 (25.8)	0.5 (0.4)

3 <sup>a</sup>data not taken.

4 <sup>b</sup>mean based on three rotorod samplings with two rods each.

1 TABLE 6. *Aspergillus flavus* communities in the soil and on crop parts in fields treated with  
2 an atoxigenic strain of *A. flavus* and adjacent to a Burkard cyclone air sampler in 1997

Month	Substrate	Propagules g <sup>-1</sup>	S strain (%)	Applied strain (%) <sup>d</sup>
June <sup>a</sup>	Soil	27,916 b <sup>c</sup>	0 l	92.7 z
	Leaves <sup>b</sup>	5,748 b	0 l	100 z
	Bolls	6,944 b	0 l	100 z
October	Soil	34,474 b	0 l	81.3 z
	Leaves	272,461 a	17.3 m	79.3 z
	Bolls	15,124 b	0 l	100 z
LSD (0.05)		218,972	1.2	25.3

3  
4 <sup>a</sup>material was sampled in late June, treatment was made in early June.

5 <sup>b</sup>leaf material was fresh leaves for the late June sample, and leaf litter resulting from chemical  
6 defoliation in October.

7 <sup>c</sup>different letters denote significantly different means (P=0.05). Means separation performed  
8 using Tukey's HSD test.

9 <sup>d</sup>percent of L strain isolates assigned to the applied VCG by vegetative compatibility analysis.

10

TABLE 7. Quantity of *Aspergillus flavus* in soils of fields surrounding two Burkard cyclone air samplers in 1998 and the incidence of both the S strain of *A. flavus* and an atoxigenic VCG of *A. flavus* applied in to the treated field in 1997

Month	Location <sup>d</sup>	Propagules <sup>a</sup> g <sup>-3</sup>		S strain (%) <sup>b</sup>		Applied VCG (%) <sup>c</sup>	
		Treated Site	Not-Treated Site	Treated Site	Not-Treated Site	Treated Site	Not-Treat Site
June	NE	491 b	1,528 b	12 nop	63 lmno	87 wx	40 wxyz
	SE	136 b	86 b	0 p	71 lm	71 wxyz	0 z
	NW	208 b	23 b	29 mnop	90 a	100 w	0 z
	SW	80 b	36 b	13 nop	44 lmnop	66 wxyz	10 yz
	Mean	229	418	14	67	81	43
October	NE	86 b	198 b	8 op	60 lmno	66 wxyz	50 wxyz
	SE	8 b	17 b	20 mnop	29 mnop	76 wxy	20 xyz
	NW	130 b	18,461 b	17 mnop	68 lmn	65 wxyz	30 z
	SW	8 b	47,120 a	3 p	90 l	55 wxyz	30 z
	Mean	58	16,449	12	62	66	33
LSD (0.05)		21,928		55.5		72.9	

<sup>a</sup>location of field sampled in relation to the Burkard cyclone sampler at that site.

<sup>b</sup>percent of *A. flavus* isolates assigned to the S strain by morphological criteria. All *A. flavus* isolates were assigned to either the S or L strains (Cotty, 1989).

<sup>c</sup>percent of L strain isolates assigned to the applied VCG by vegetative compatibility analysis.

<sup>d</sup>different letters denote significantly different means (P=0.05). Means separation performed using Tukey's HSD test.

Fig. 1. The Burkard cyclone sampler (Burkard Manufacturing Co., Rickmansworth, UK) used in this study.

Fig. 2. The location of the two Burkard cyclone samplers in relation to fields treated with the atoxigenic VCG of *Aspergillus flavus* in the Mohawk Valley, Arizona.

Fig. 3. The rotorod sampling apparatus developed for this study showing the construction of tubular PVC piping with motors inserted in joint fittings.

Fig. 4. Characteristics of samples collected by Burkard cyclone samplers at a site treated with an atoxigenic strain of *Aspergillus flavus* and at an untreated site: (A) weight of sampled material; (B) quantity of total fungi, *A. flavus*, and *A. niger*; (C) proportion of total fungi consisting of *A. flavus*; (D) proportion of *A. flavus* in relation to *A. niger*; and (E) quantity of bacteria. \* indicates missing data points.

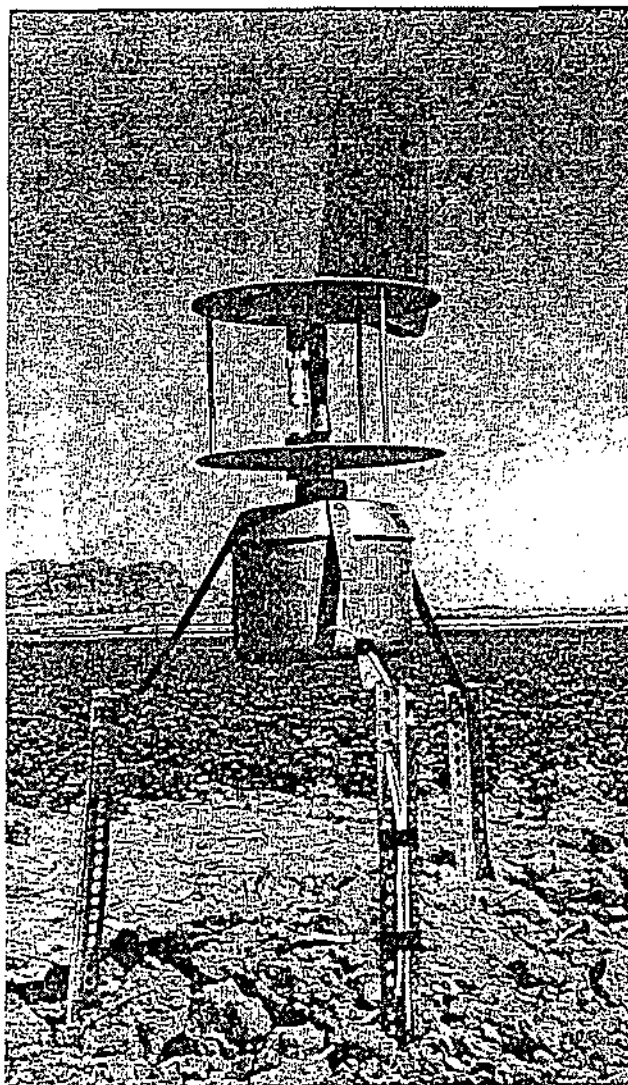
Fig. 5. Characteristics of *A. flavus* communities sampled by Burkard cyclone samplers at a site treated with an atoxigenic vegetative compatibility group (VCG) and at an untreated site: (A) proportion of *A. flavus* consisting of the L and S strains; (B) proportion of the L strain consisting of the applied atoxigenic VCG. \* indicates missing data points.

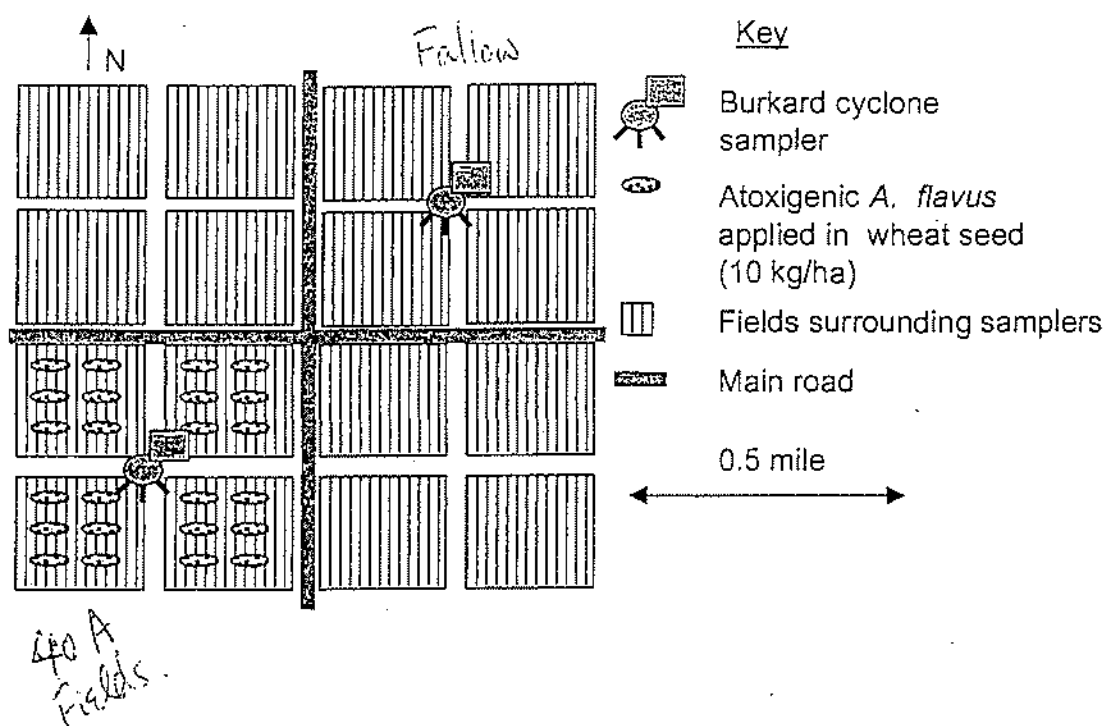
Fig. 6. Weather data for Roll, AZ, from 1997-1999.

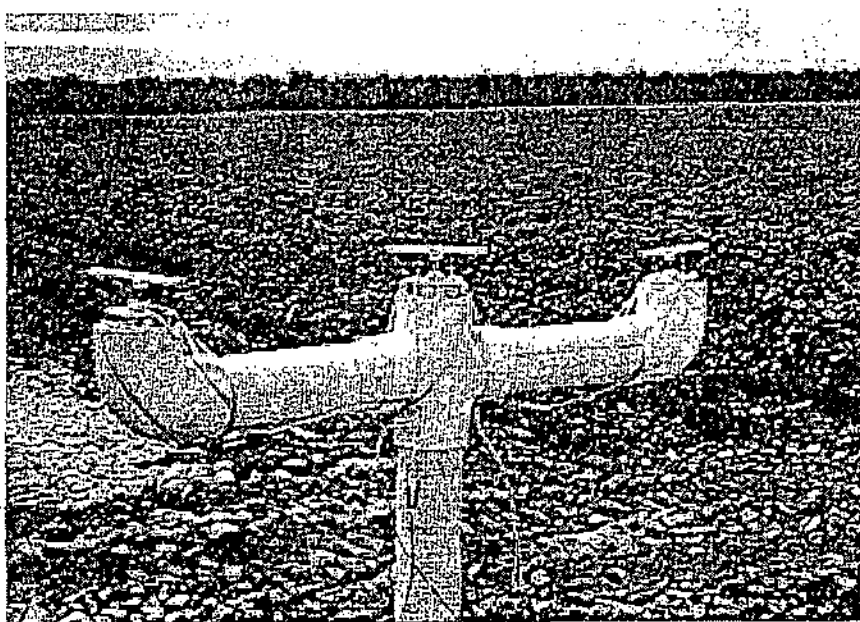
Fig. 7. Frequency of different size particles sampled by the Burkard cyclone sampler.

Fig. 8. Passage of *Aspergillus flavus* propagules sampled by the Burkard cyclone sampler through membrane filters with different pore sizes.

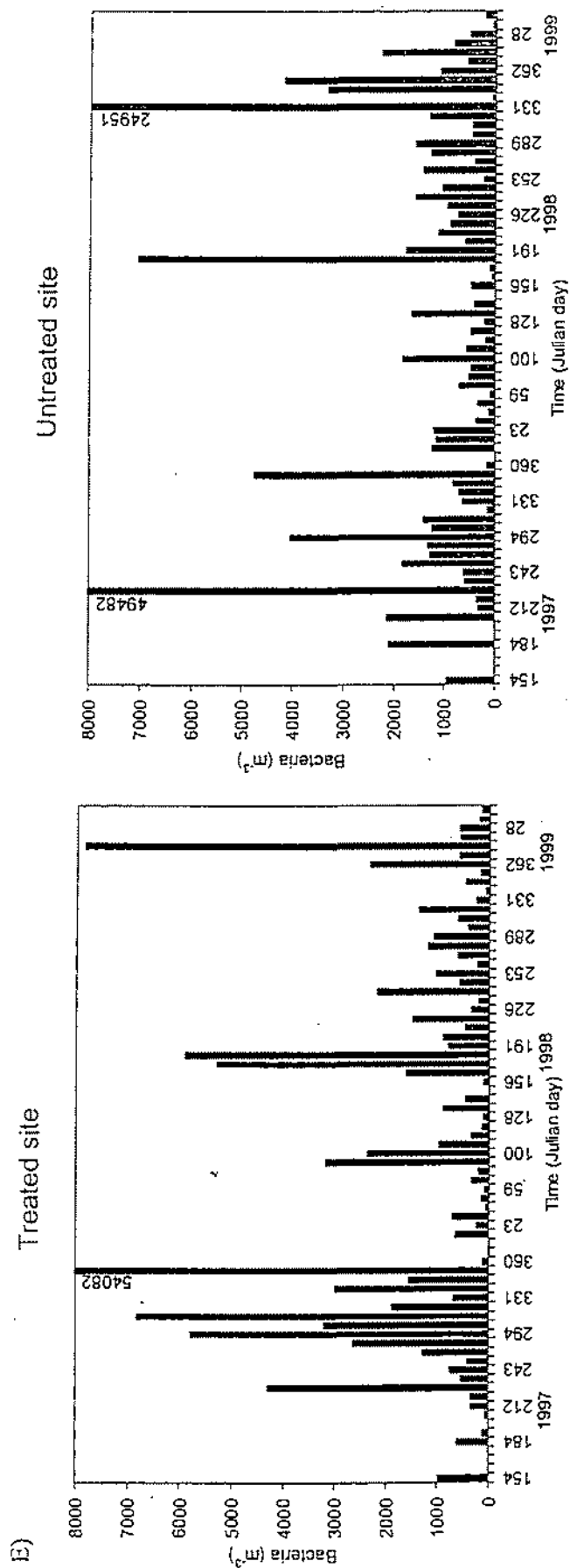
Fig. 9. The results of laboratory tests using rotorods coated with either silicone grease or carboxymethylcellulose/water/glycerol to sample conidia of *Aspergillus flavus* (A) numbers of conidia sampled, (B) the frequency of encounters with conidia, and (C) the range of cluster sizes of conidia sampled.



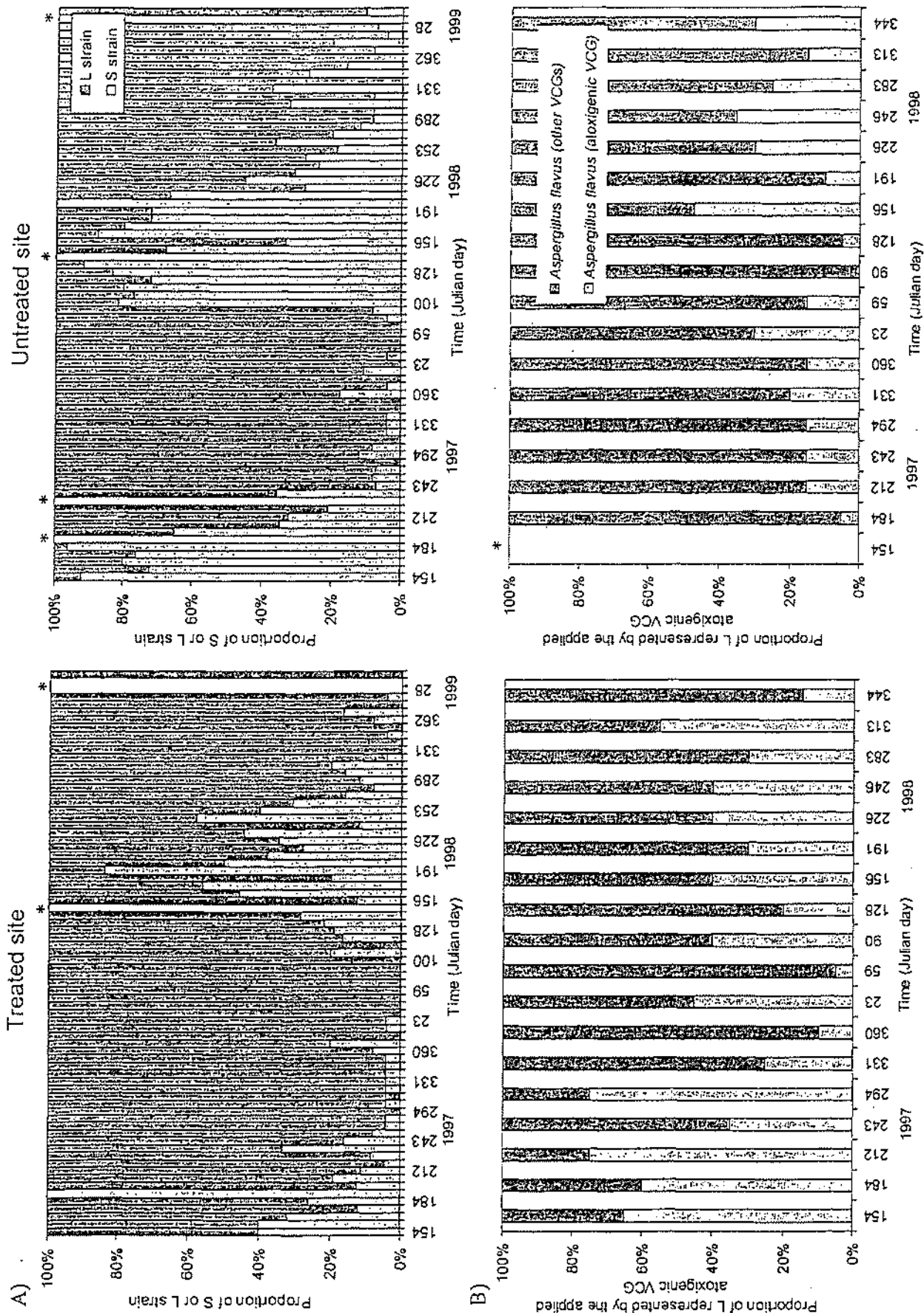


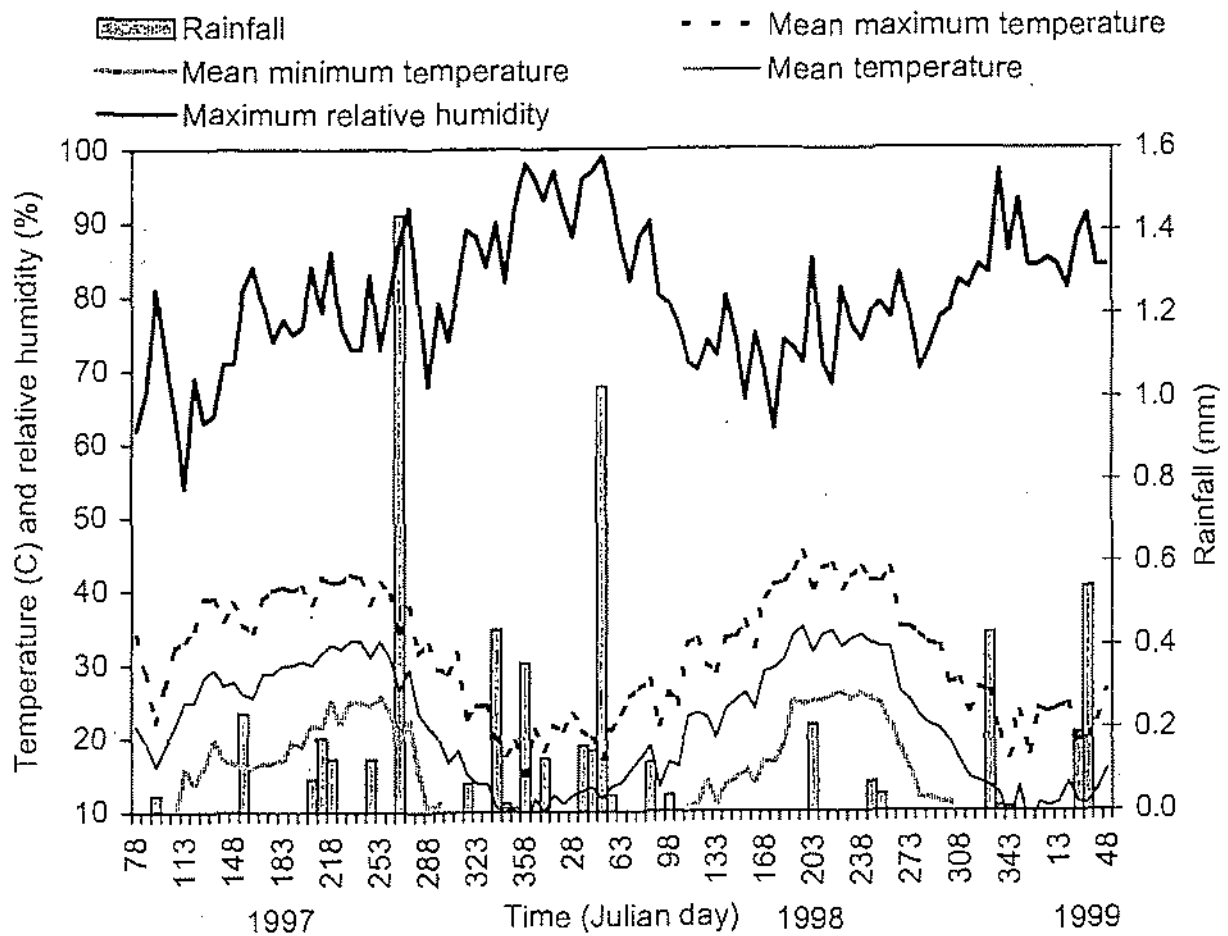


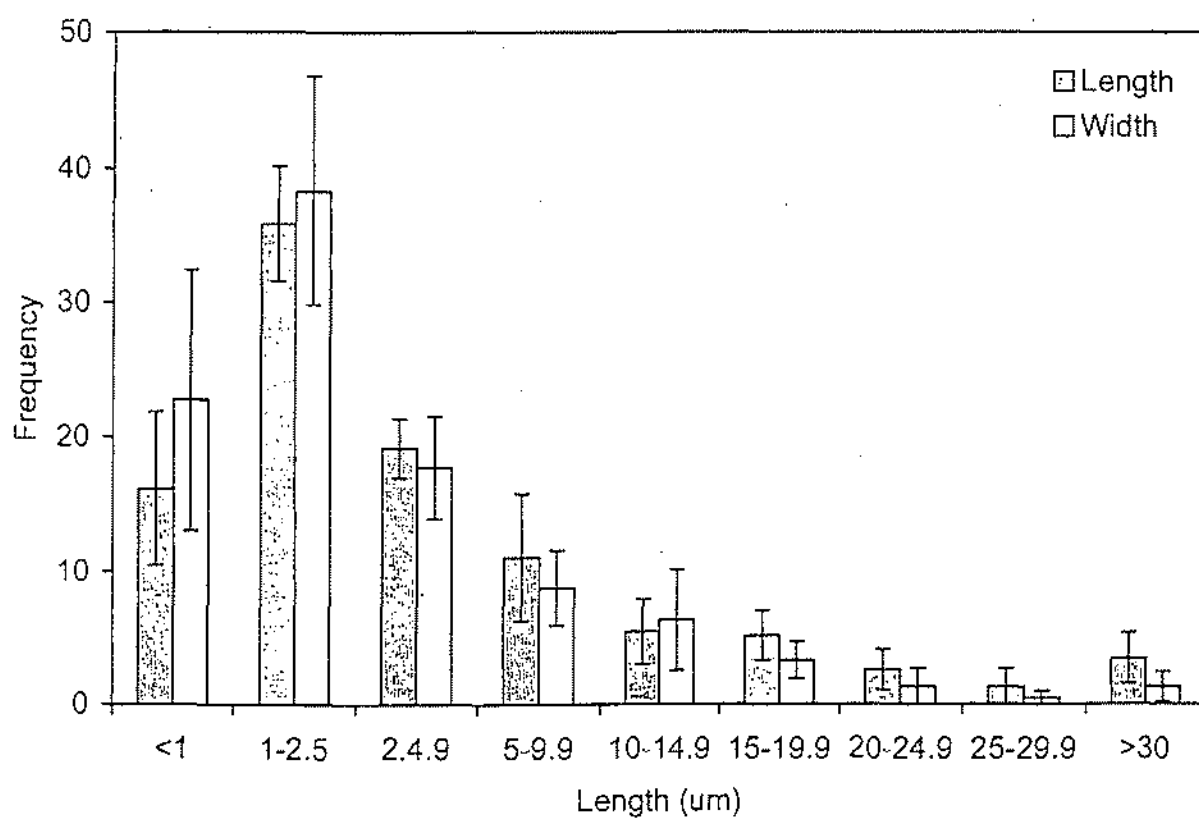
Epidemiology of *Aspergillus flavus* in Arizona, Figure 4, cont.

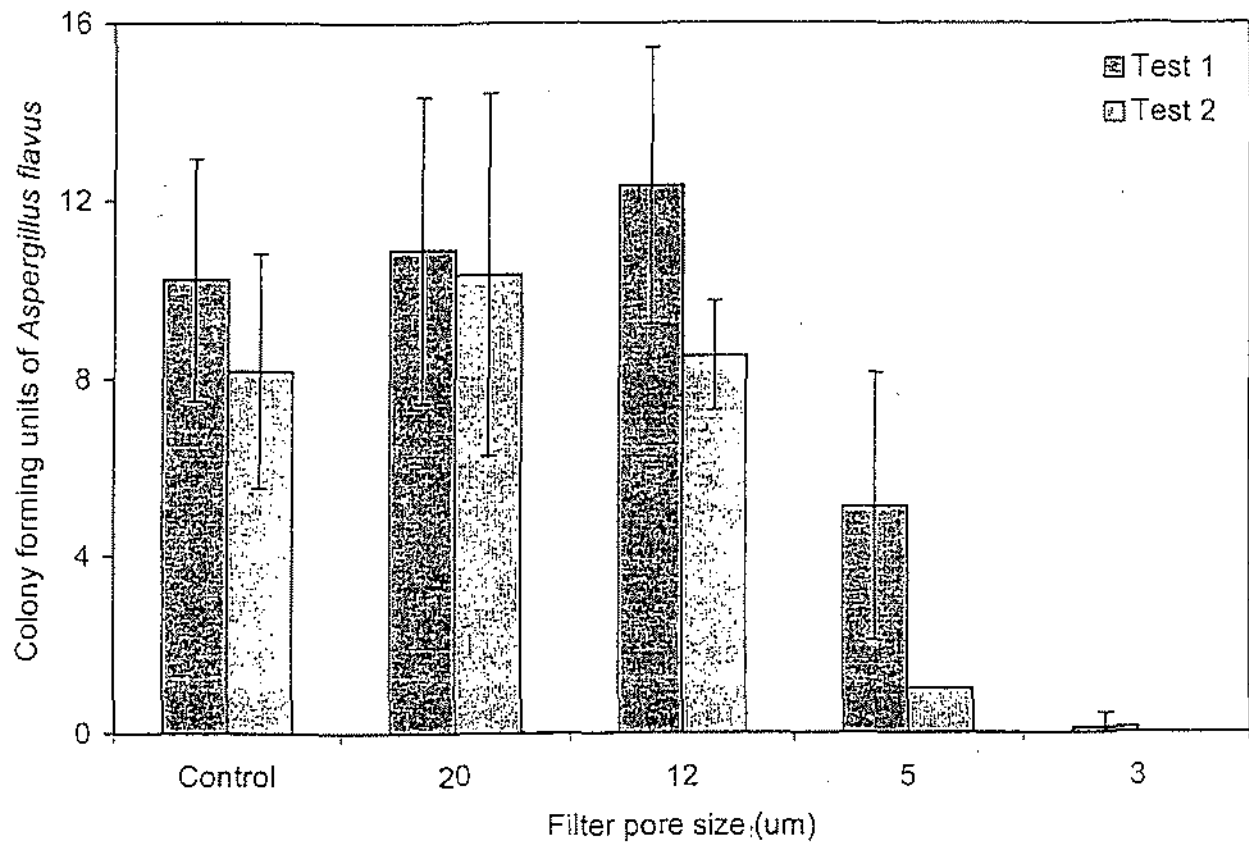


Epidemiology of *Aspergillus flavus* in Arizona, Figure 5

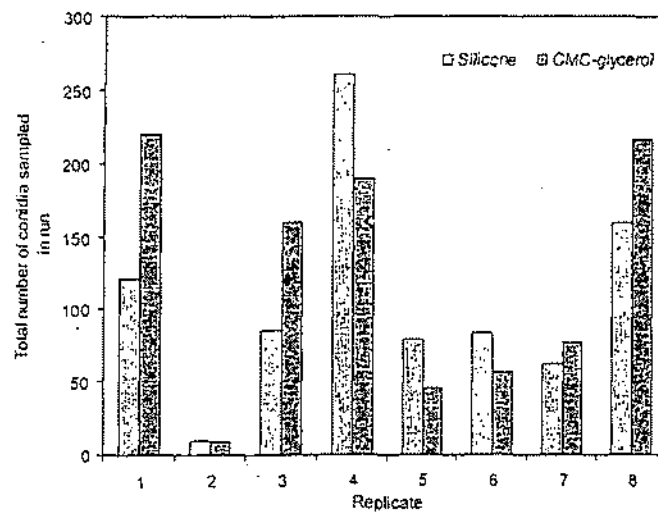




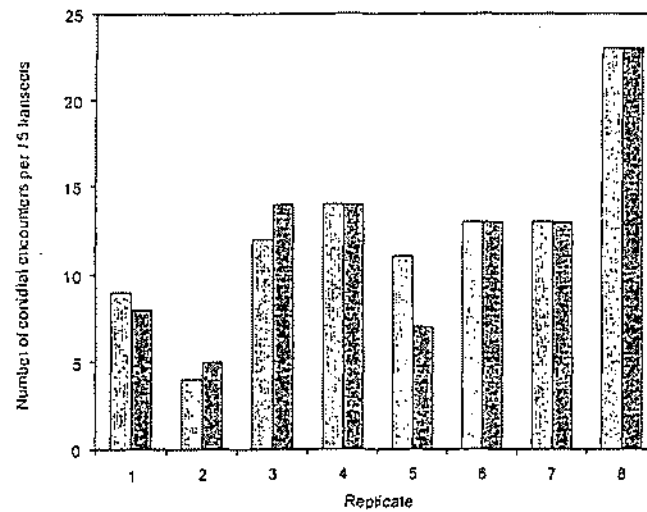




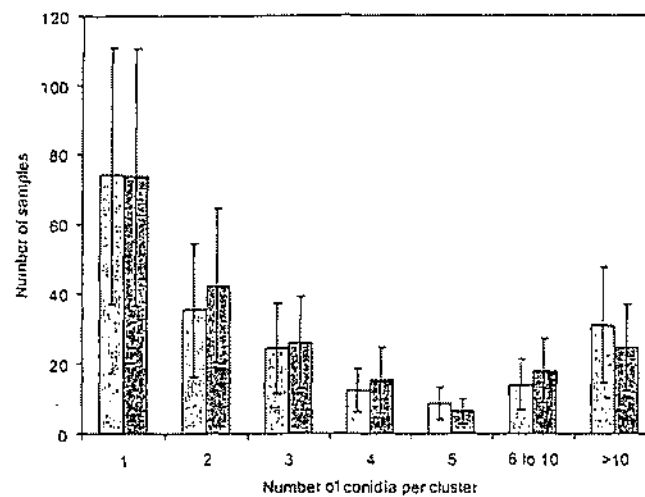
(A)



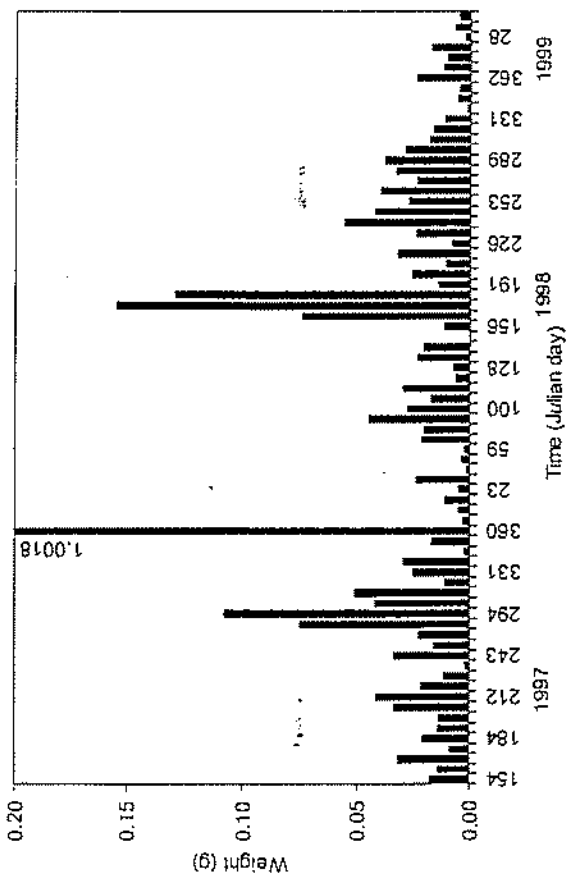
(B)



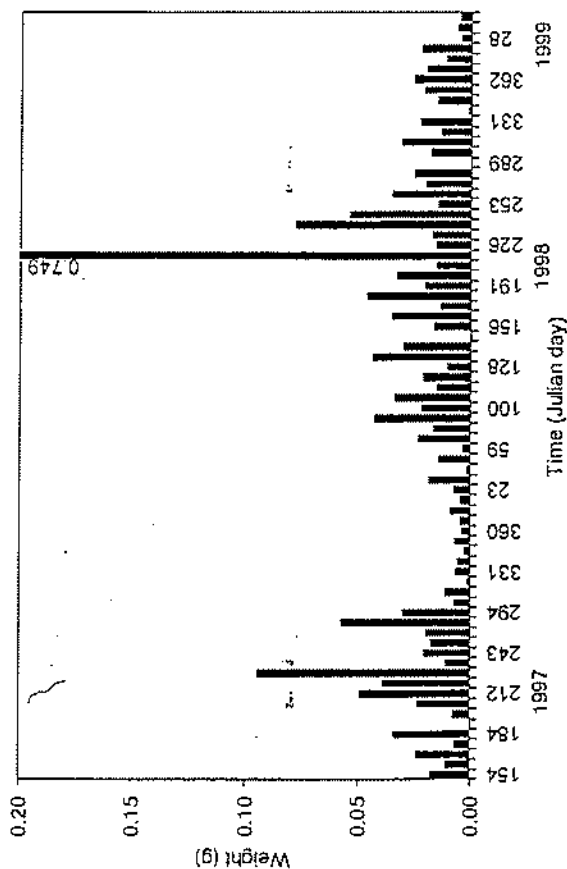
(C)



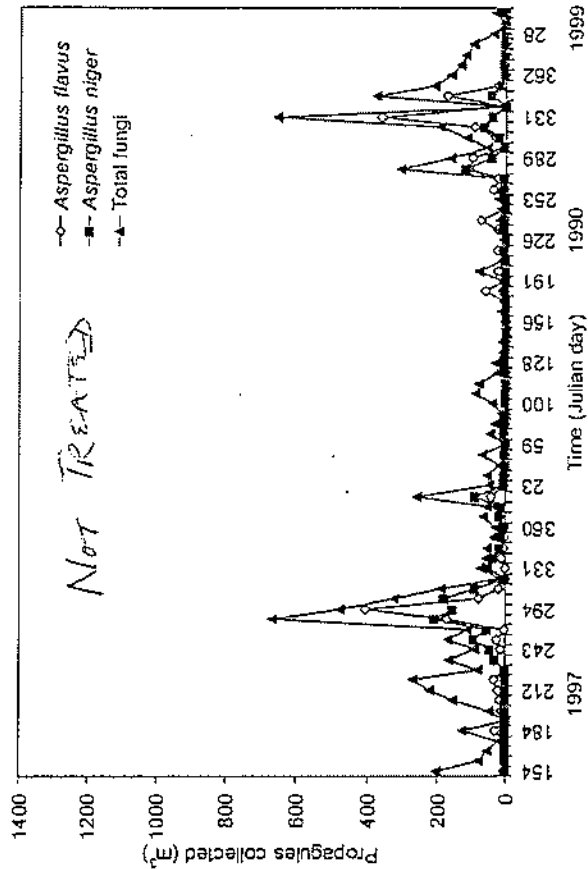
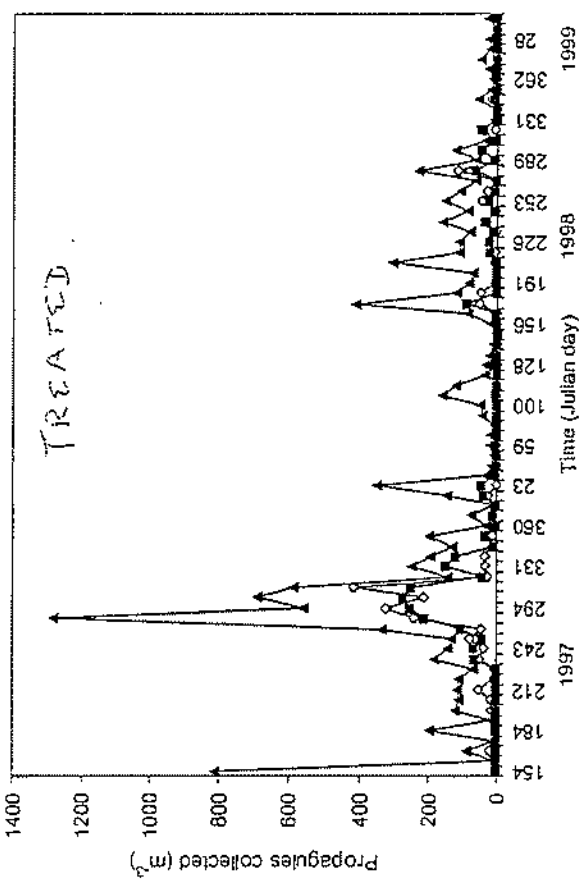
A) Treated site



Untreated site



B)

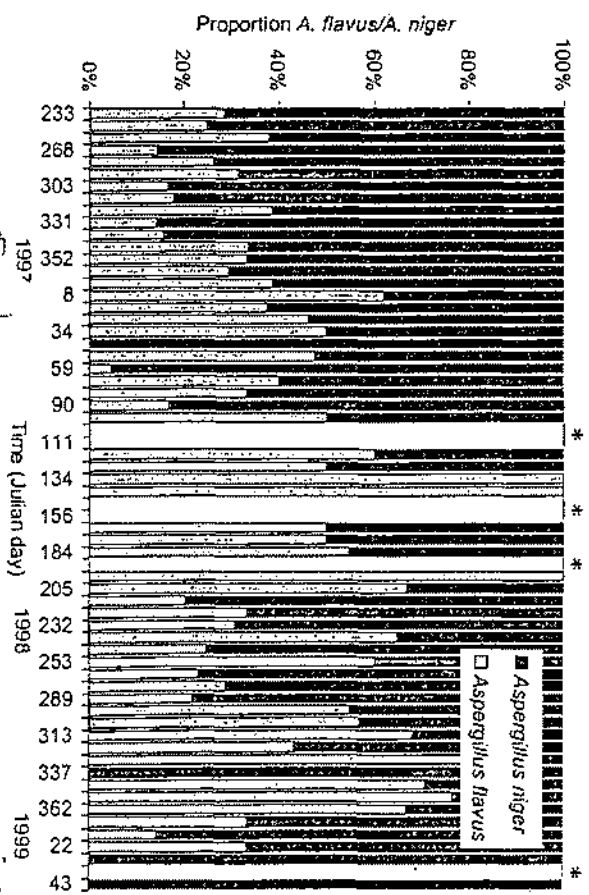
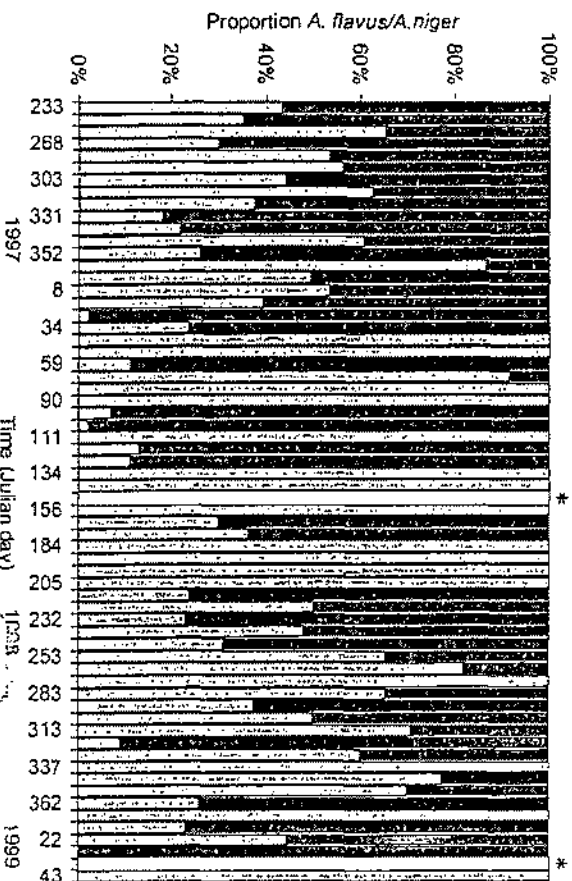
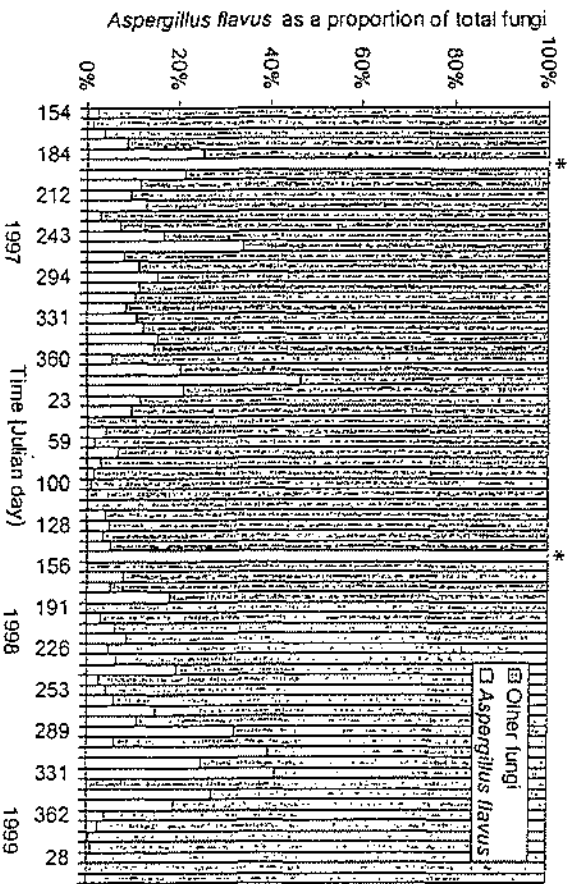
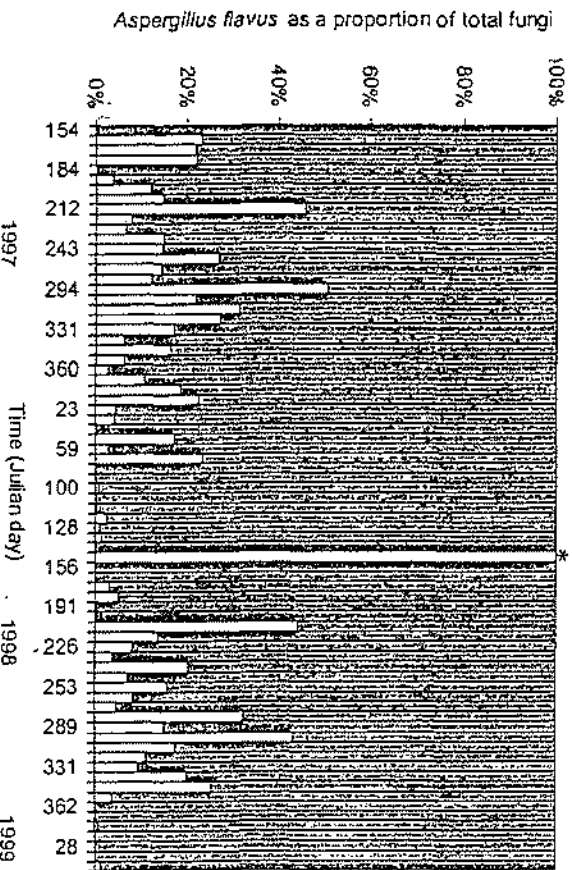


C)

Treated site

D)

Untreated site



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## The ARS-ACRPC Partnership to Control Aflatoxin in Arizona: Current Status

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In 1998 the Arizona Cotton Growers Association voted to establish a grower owned manufacturing facility to provide sufficient quantities of inoculum of the atoxigenic *Aspergillus flavus* strain, AF36, for statewide treatment of Arizona cotton. The Arizona Cotton Research and Protection Council (ACRPC) and the USDA Agricultural Research Service (ARS) initiated a partnership. Goals included: (1) Development of management strategies; (2) Optimization of application and agronomic practices to achieve short and long-term aflatoxin reduction in cottonseed; (3) Development of processes to be used by growers to produce needed quantities of inoculum; and (4) Compilation of information for full Environmental Protection Agency (EPA) registration of AF36. In 2001, the 3rd year of the collaboration, 19,975 acres were treated in four areas extending from the far western edge of Arizona to the south central portion of the state.

In 2000 four areas were treated. The total treated acreage was 16,094. *A. flavus* communities present on crops and in soils were characterized in treated and control areas in order to assess the extent to which community structure had been altered by applications. Over 6,000 isolates were classified by strain identification and vegetative compatibility. Treatment areas were separated from non-treated control fields by one mile buffer zones. Soil, crop seed and air samples provided the basis for program analyses. Toxin test results were requested from commercial gins and cottonseed wholesalers and, on a limited number of fields, independent sampling and analysis were performed to confirm the commercial tests. Incidences of AF36 showed variable but positive results throughout all treatment regions. Evidence of treatment influences in control areas from treatments in 1999 and 2000 was also detected. In the Roll/Wellton area, 80% of the *A. flavus* on the crop after ginning was AF36 versus 27% on untreated control fields. Incidences of AF36 were 52-72% on the ginned crop in eastern and northwestern Paloma and 93% in the southwestern portion. The southwest fields were treated by ground with material banded under the canopy. Other portions of Paloma were broadcast by air. Untreated Paloma controls ranged from 11-27% AF36 crop incidence. Northern Maricopa County fields produced treated versus non-treated crop AF36 incidences of 30% vs 5% (Laveen) and 90% vs 3% (Peoria). In the Maricopa-Stanfield area of Pinal County AF36 crop incidences of 76% (Marathon Farms) and 71% (Ak Chin Farms) compared favorably to an 11% average in untreated controls. The most precise aflatoxin results came from this Maricopa-Stanfield area. Working closely with a key grower and gin personnel, commercial toxin tests were run on seed piles from individual fields comprising a large contiguous block of cotton. Fourteen of seventeen fields tested (82%) were below 20 ppb. All control seed lots were over 100 ppb. According to the grower, the farm had never previously produced clean seed.

The manufacturing process has increased to production of 1,200 pounds per batch. During 2001 design changes were made to the process and the required equipment is being fabricated. It is anticipated that the ARS-ACRPC facility will be able to manufacture 6,000 lb of AF36 material per day by the end of 2002. Registration of *Aspergillus flavus* AF36 with the USEPA limited 2001 treatments to fewer than 20,000 acres. In 2001 additional safety, environmental, and efficacy data were provided to EPA. Additional tests required including avian and mammalian toxicity tests. It is anticipated that these will be completed by early 2002. Pending the results of those tests, full registration of AF36 on cotton in Arizona will be aggressively pursued.



## Wheat Seed Colonized with Atoxigenic *Aspergillus flavus*: Characterization and Production of a Biopesticide for Aflatoxin Control

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Biocontrol of aflatoxin contamination using atoxigenic *Aspergillus flavus* to competitively exclude aflatoxin-producing strains has previously been reported, and is currently in the third year of commercial-scale tests (treating 50-200 ha per annum). Wheat seed colonized with atoxigenic *A. flavus* has been used in the commercial trials. Requirements for production of this colonized wheat seed are described and the spore yield of wheat is compared to other substrates. The study suggests that the most cost-effective inoculum production would require colonization of wheat with  $10^{10}$  conidia  $kg^{-1}$  of wheat seed at 25% wheat moisture for 18 h at 31°C. To prevent fungal growth and associated wheat aggregation in storage, seed had to be dried below 15% wheat moisture, although a moisture content of 18% wheat did not reduce viability in sealed containers stored at 18-25°C over an 8-month period. The dry biopesticide had multi-year stability without refrigeration and withstood temperatures of 70°C for 20 min. Sporulation of the product occurred within 1 days at 31°C and 100% relative humidity with yields averaging  $4.9 \times 10^8$  conidia  $g^{-1}$  by day 7.

**Keywords:** *Aspergillus flavus*, aflatoxin, biopesticide, formulation

### INTRODUCTION

Aflatoxins are extremely toxic mycotoxins produced by some members of *Aspergillus* section *flavi*. These fungi are ubiquitous and infect many crops and crop products including peanuts, corn, cottonseed and a variety of nuts (Diener *et al.*, 1987; Cotty *et al.*, 1994). In order to keep the aflatoxin content of foods and feeds below potentially dangerous levels, maximum permissible aflatoxin contents are mandated in most countries. Produce with aflatoxin exceeding these limits has reduced value. Both health risks and the reduced profitability of contaminated crops create a need to prevent the formation of aflatoxins (Park *et al.*, 1988).

Affected material may be decontaminated using ammonia (Park *et al.*, 1988). The process

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is costly, not universally available, and ammoniated crops generally have reduced value. Methods to reduce aflatoxin formation include manipulation of harvest date (Bock & Coity, 1999), irrigation practice (Russell *et al.*, 1978), harvest method (Russell *et al.*, 1981) and storage practice (Barnes *et al.*, 1997).

Biocontrol of aflatoxin-producing strains with atoxigenic strains of *A. flavus* is being developed on corn (Brown *et al.*, 1991), cottonseed (Coity, 1994) and peanuts (Dorner *et al.*, 1992). Atoxigenic strains are used to competitively exclude aflatoxin producing strains during crop colonization (Coity & Bayman, 1993; Coity *et al.*, 1994). Atoxigenic strains have been applied with rice kernels and wheat seed, in alginate pellets and in conidial suspensions (Dorner *et al.*, 1992; Daigle & Coity, 1995; Coity, 1994). When applied as a solid formulation, the biocontrol agent is activated by moisture from irrigation, rainfall or dew and produces conidia which are dispersed to the crop.

For commercial use, formulations of biocontrol agents must allow cost effective production and adequate stability to allow the product to be transported, stored, and applied in a commercially compatible manner without significant loss in viability. Coity (1994) initially used colonized wheat seed as a source of atoxigenic *A. flavus* in field plot experiments. Daigle and Coity (1995) experimented with various forms of alginate pellet and found that those containing wheat gluten and mycelia of atoxigenic *A. flavus* produced more conidia than wheat seed ( $4.0 \times 10^8 \text{ g}^{-1}$  vs  $1.0 \times 10^8 \text{ g}^{-1}$  after 7 days incubation). However, alginate pellet production is a multi-stage process requiring constituents that are expensive compared to non-processed wheat (Daigle & Coity, 1997). The cost of the bulk product for alginate pellets was estimated to be US\$2.53–5.16  $\text{kg}^{-1}$  while the cost of wheat is US\$0.18–0.26  $\text{kg}^{-1}$ . Inherent difficulties and expense of producing sufficient alginate pellets for large-scale field trials hampered further testing. New methods for the production of alginate pellets may help reduce the cost of encapsulating biocontrol agents (Daigle *et al.*, 1997; Daigle *et al.*, 1998). However, the simplicity and field success of colonized wheat has led us to develop that alternative.

Since 1989, the wheat seed formulation has been empirically improved, and large scale field trials (200 ha in 1997 and 1998) directed at preventing aflatoxin contamination of cottonseed have been undertaken, using this product as the sole inoculum source (Coity, 1997). These trials have been strongly supported by the agricultural community in Arizona (Anon., 1996a, b; Rayner, 1998). The process for colonizing wheat with *A. flavus* has been incrementally modified and the current process, used to produce 2200 kg of colonized wheat in a laboratory in two months, has only been repeated in materials submitted to the United States Environmental Protection Agency (EPA).

Any method used to control an agricultural problem must fit the economics of the production system. The following paper outlines the production of an inexpensive and stable biopesticide intended for use in preventing aflatoxin contamination. Experiments were undertaken to characterize better the colonized wheat used in commercial field tests currently underway, while optimizing and refining the process used to produce this product.

## MATERIALS AND METHODS

### General Methods and Protocol

**Inoculation, distribution and drying of material.** A standard process for manufacturing *A. flavus* colonized wheat was allowed by the EPA under Experimental Use Permit 09224-EUP-R. In that procedure, 1 kg of hard red winter wheat seed (Organic Grade, Arrowhead Mills, Hereford, TX, USA) was added to 2 l Nalgene plastic containers (Nalgene Labware, Rochester, NY, USA) with 70 ml of water. The wheat seed-containing bottles were rolled for 20 min, to allow even distribution of the moisture and autoclaved for 60 min. After setting overnight at room temperature, the wheat seed was autoclaved for a second 60 min. After cooling, the wheat seed was ready for inoculation. *A. flavus* strain AF36 (Coity, 1994)

was cultured on V8 juice agar (5% V8 juice, 2% agar, pH 5.5) in Petri dishes. Conidia were harvested from 7-day-old cultures with sterile swabs and suspended in sterile distilled water. Conidial concentrations were measured using a turbidity meter (Orbicon-Hellige Digital Direct-Reading Turbidimeter, Orbicon Analysis Systems Inc., New York, USA). A linear nephelometric turbidity unit (NTU) vs colony forming unit (CFU) standard curve was developed to relate turbidity to conidial concentration. Spore suspension (150 ml,  $1.1 \times 10^7$  conidia  $\text{ml}^{-1}$ ) was added to each canister and the canisters were rolled horizontally for 3 h at 3 rpm on a tissue culture roller (Modular Cell Production Roller Apparatus, Wheaton, Millville, NJ, USA) to allow even absorption of the liquid and even dispersal of the inoculum. After rolling, canisters were incubated static at 31°C for 24 h. At initiation of incubation, the wheat moisture ranged from 28–32% (w/w) depending on the moisture content of the starting grain. Moisture was determined using an Ohaus Moisture Determination Balance (Model MB200, Ohaus Corporation, Florham Park, NJ, USA). After incubation, the seed was placed in sterile cotton pillowcases (72.5 cm x 47.5 cm, seven canisters per bag) and placed in a forced-air tray oven (Proctor & Schwartz, Inc., Philadelphia, PA, USA) at 58°C to dry for 48 h. Incoming air was ducted through a HEPA-filter. Seed was then transferred to 19 l polyethylene food containers (Letica Corp, Rochester, MI, USA) for storage and transport.

A series of tests were performed to optimize the above procedure and to characterize the resulting product. Unless otherwise stated, treatments were replicated three times, and the experiments were repeated twice. All experiments were fully randomized.

**Spore yield.** The spore yield of the colonized wheat was quantified by placing one seed in each cell of a sterile 12-cell multi-well plate (Corning Glass Works, Corning, NY, USA). Replicates consisted of one multi-well plate (12 cells). Each treatment was replicated three times. Water (10–12 ml) was added only in the inter-cell spaces; plates were covered, placed in sealed plastic containers to prevent evaporation and transferred to a water-jacketed incubator at 31°C. Spore yield was measured on four seed randomly selected from each plate. The seed were placed in a 100-ml bottle containing 50 ml of 70% aqueous ethanol. Conidia were dislodged by agitation (10 s) and the turbidity of the solution measured by turbimetry as described previously. The spore concentration was extrapolated from the NTU/CFU standard curve. Tests showed that wheat seed alone did not contribute significantly in the NTU of the spore suspensions measured. After considering the kinetics of sporulation in this system, 7 days was chosen as the standard incubation period for spore yield assessment. The percentage of seeds with sporulation was also recorded for each assessment.

**Visual assessment of colonization.** For some experiments, the seed surface colonization was assessed visually. Ten seeds were fixed in an acetic acid:ethanol (50:50 v/v) fixative and stained with 0.5% methylene blue in lactophenol. Seed were examined with a dissecting microscope (X63) with oblique illumination. Fungal colonization of the dorsal (upper) and ventral or crease (lower) surfaces was scored separately using a 1–5 scale: 1 = no colonization visible; 2 = 1–5 mycelial strands visible; 3 = 6–20 mycelial strands visible; 4 = 21–50 mycelial strands visible and 5 = > 50 mycelial strands colonizing the surface.

#### Effect of Concentration of Conidia on Colonization of Wheat Seed

Influence of initial spore concentration on colonization of wheat seed was assessed by altering the concentration of conidia used in the standard procedure. Evaluated concentrations included  $0$ ,  $10^2$ ,  $10^3$ ,  $10^4$ ,  $10^5$ ,  $10^6$ ,  $10^7$  or  $10^8$  conidia  $\text{kg}^{-1}$  of wheat seed.

#### Incubation Period

In order to optimize the required incubation period, subsequent to rolling for 3 h, the inoculated wheat seed was placed at 31°C for 0, 4, 8, 18, 24 and 48 h. After incubation, seed was dried according to the standard procedure and spore yield was quantified. Colonization

was visually assessed. To assess further the degree of colonization, seed from the 0, 8, 24 and 48 h incubation periods were surface disinfected with 70% ethanol for 0, 15, 30, 60, 120 and 180 min. After ethanol treatment, seed were washed twice in sterile distilled water and fungal growth was visually assessed and spore yield quantified after 7 days (100% relative humidity 1RH, 31°C) as described previously.

#### Effect of Moisture Content on Colonization of Wheat Seed

The moisture content required for successful product manufacture was determined by adjusting wheat moisture during incubation to either 10, 12, 15, 20, 25, 30 or 35% (w/w). The desired moisture content was produced by adding different quantities of water to wheat seed that had been pre-sterilized and dried. After the standard sterilization process, the seed were dried to 3% (w/w) moisture in the forced air oven at 80°C. A moisture content of 10% (w/w) was obtained by adding 70 ml of spore suspension (comprising 50 ml spore suspension,  $1.0 \times 10^6$  conidia ml<sup>-1</sup> plus 20 ml water) to 1 kg of dried wheat. The volume of water was adjusted to achieve target moistures. After 24 h incubation (31°C), seed was dried according to the standard procedure and colonization was visually assessed. Colonization was also assessed by surface disinfection of seed samples from each treatment with 70% ethanol for 20 min (shown to be an effective time period in the previous experiment). The seed was washed twice in sterile distilled water and fungal growth was visually assessed after 7 days as previously described.

#### Effect of Seed Substrate on Spore Yield

Wheat seed, red sorghum, black-eyed peas, black beans, soy beans, barley, rye, oats, corn, Pima cottonseed, finger millet seed and rice grains were compared for ability to support conidial production by *A. flavus*. The standard procedure was followed for inoculation, incubation, drying and quantification of spore yield. For each substrate, 100-grain weights were measured and used to calculate spore yield g<sup>-1</sup>.

#### Stability and Viability of the Wheat Seed/*A. flavus* formulation

**Effect of time.** Colonized wheat seed manufactured by the standard process was stored in plastic bags (Quari Size, Ziploc, Duw Brands L.P., Indianapolis, IN, USA) at room temperature from 12 May 1995–15 October 1997. Viability and spore production were assessed at approximately monthly intervals over this 29-month period. Seed were randomly selected from an individual bag on each occasion. Spore yield was quantified using the procedure already described for incubating seed in multi-well plates. However, on each occasion five NTU measurements were made on individual seeds.

**Effect of moisture content and drying period.** To assess the impact of drying period on product performance, colonized wheat seed produced by the standard process was dried in a forced air oven for 0–138.5 h. At each sampling time, moisture content, viability and spore yield were determined. In order to assess the impact of moisture content on stability and viability, at each sampling time a 50 g subsample was sealed in an air-tight polypropylene container. Samples were stored at room temperature (18–25°C) for 8 months and subsequently assessed for viability and spore yield.

**Effect of heat.** Heat tolerance was assessed by heating colonized wheat seed to either 60, 70, 80, 90, 100 or 110°C for 20 min. Heating was performed in 5 ml sterile glass vials (with loose caps) containing 20 seed each. In a second experiment, seed was heated for 20 min at either 70, 74, 76, 78, 80, 82, 84, 86 or 90°C. In a third experiment, colonized wheat was heated to 80°C for either 0, 5, 10, 20, 40 or 80 min. In all experiments viability of the biopesticide was tested after treatment by incubating seed in multi-well plates as previously described. Each experiment was replicated three times and was performed twice. In order to

determine the actual heat exposure of the product during on farm storage in western Arizona, temperature dataloggers (HOBO-Temp, Onset Computer Corporation, Piquasset, MA, USA) were placed in wheat containers shipped to Arizona for use on the 1997 and 1998 crops. The biopesticide, *A. flavus* AF36 (EPA Registration No. 69224-EUP-1), is labelled for packaging in 19 l polyethylene food containers. Dataloggers were placed in plastic bags within the packaged product and thus were exposed to the same conditions as the product to the point of application in farmer's fields.

#### Data Analysis

SAS (SAS Institute Inc., SAS Campus Drive, Cary, NC, USA) was used to analyze the data. Analysis of variance (ANOVA) was applied to all mean comparisons and mean separations were performed using Tukey's HSD test ( $P=0.05$ ). Regression analysis was used to investigate and describe the relationships between different variables (effects of drying period on moisture content, temperature on viability, storage period on viability, and incubation period on sporulation). Prior to analysis, spore yield data from the substrate-type tests were square root transformed to reduce heterogeneity of variance between treatments.

## RESULTS

### Spore Production on Wheat Seed Colonized by *A. flavus*

At 31°C, significant numbers of spores ( $5.4 \times 10^8$  conidia  $g^{-1}$ ) were produced within 3 days (Figure 1) and the quantity produced rapidly increased from 4–7 days. Thereafter, the rate of spore production slowed until the test was terminated at 14 days ( $6.5 \times 10^8$  conidia  $g^{-1}$ ).

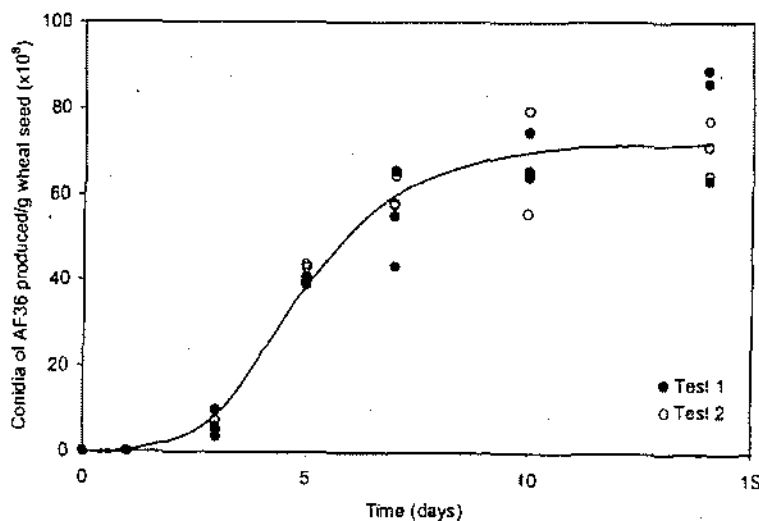


FIGURE 1 Sporulation of *A. flavus* strain AF36 on colonized wheat seed after different periods of incubation. The regression solution is a Gompertz sigmoidal model,  $f = e^{(4.23x - 1.1355(e^{4.23x}))}$ ,  $R^2 = 0.98$ .

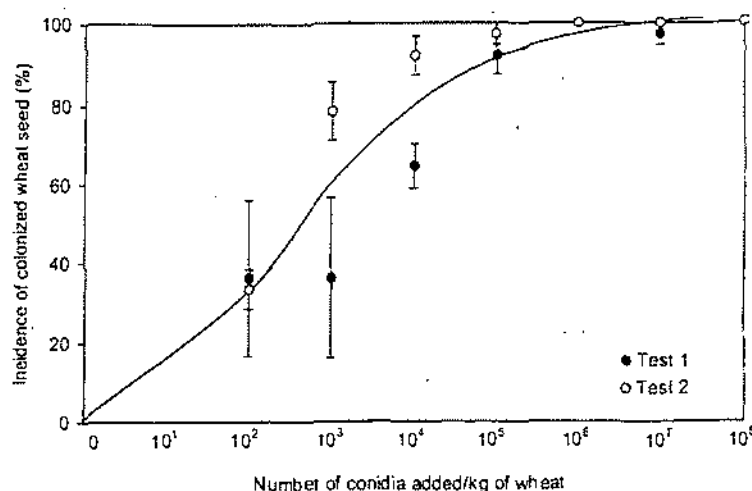


FIGURE 2 Effect of spore concentration of *A. flavus* strain AF36 on wheat seed colonization. Regression solution is a Gompertz-sigmoidal model,  $y = e^{(0.05 - 4.00004x)}$ ,  $R^2 = 0.85$ .

#### Effect of Concentration of Conidia on Colonization of Wheat Seed

Increased conidial concentration resulted in higher incidence of colonized wheat seed (Figure 2). Colonization increased significantly with greater conidial concentration up to  $10^5$  conidia  $\text{kg}^{-1}$ . Increases in colonization above 97% were not significant (Tukey's HSD,  $P = 0.05$ ).

#### Effect of Incubation Period on Colonization of Wheat Seed

Initial evaluations suggested incubation period does not influence the extent of wheat colonization because 100% of the evaluated seed from all incubation periods produced statistically similar ( $P < 0.05$ ) spore yields ( $1.5\text{--}12.5 \times 10^8$  conidia  $\text{g}^{-1}$  wheat seed). However, microscopic examination of the surface indicated an influence of incubation period on seed surface colonization (Table 1). Wheat incubated for 18–48 h had greater ( $P = 0.05$ ) colonization on the dorsal surface than wheat incubated for 0–8 h, and colonization of the ventral surface was greater than on the dorsal surface at 4 and 8 h. At time 0, colonization did not exceed uninoculated controls and no fungal mycelia were observed on thral surfaces. At 18-h incubation or more, the two surfaces had similar levels of colonization. Surface disinfection of the wheat seed also indicated that seed incubated for 24 h or more was better colonized by *A. flavus* than seed incubated for 8 h or less (Figure 3). A low percentage (5–10%) of seed colonized for 24 or 48 h retained viable *A. flavus* after washing in 70% ethanol for 3 h.

#### Effect of Moisture Content on Colonization of Wheat Seed

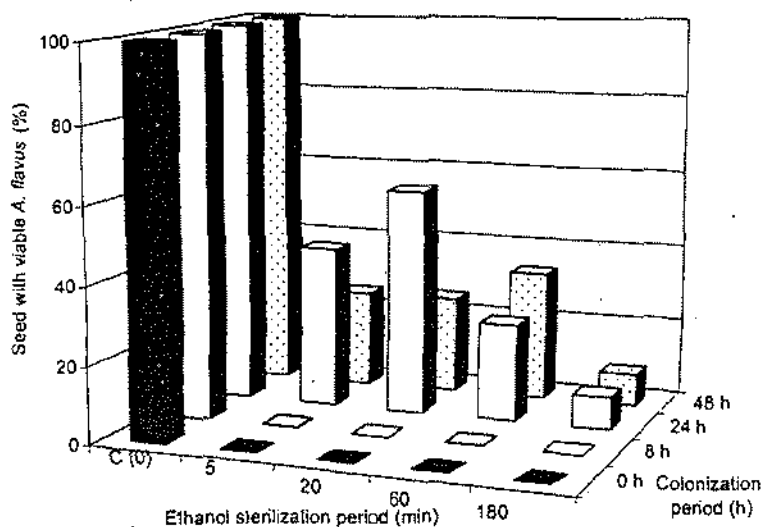
Microscopic examination revealed significant ( $P < 0.05$ ) differences in the amount of fungal growth on seed incubated with different moisture contents (Table 2). Below 25% moisture, fungal growth was sparse on both seed surfaces. At 25% moisture and above, mycelial growth was dense. Similarly, the surface disinfection with 70% ethanol showed greatest reduction in survival when seed was colonized at 20% moisture or less (Figure 4).

TABLE 1. Effect of incubation period on wheat seed colonization by *A. flavus* strain AF3b

Incubation period hr	Colonization (visual assessment scale 1-5) <sup>a</sup>	
	Upper surface	Lower surface
Control <sup>b</sup>	1.0 a	1.8 a
0	1.0 ab	1.8 a
4	1.3 b	2.6 a
8	1.9 b	4.3 b
18	4.9 c	4.6 b
24	4.7 c	4.6 b
48	3.1 c	4.8 b

<sup>a</sup>Numbers in a column followed by a common letter are not significantly different using Tukey's HSD test. Fungal colonizations of the dorsal (upper) and ventral or crease (lower) surfaces were scored separately using a 1-5 scale where 1 = no colonization visible; 2 = 1-5 mycelial strands visible; 3 = 6-20 mycelial strands visible; 4 = 21-50 mycelial strands visible and 5 = > 50 mycelial strands visible.

<sup>b</sup>Control not inoculated.

FIGURE 3. Effect of disinfection for various periods with 70% ethanol on survival of *A. flavus* strain AF3b in sterile wheat colonized for different periods at 21°C.

#### Effect of Seed Substrate on Spore Yield of *A. flavus*

Seed differed significantly ( $P < 0.001$ ) in ability to support spore production (Table 3). Both wheat ( $11.4$  and  $7.2 \times 10^9$  conidia  $g^{-1}$  in tests 1 and 2, respectively) and oats ( $7.6$  and  $9.2 \times 10^9$  conidia  $g^{-1}$  in tests 1 and 2, respectively) gave consistently high spore counts in the two tests. Pima cottonseed ( $0.8$  and  $1.7 \times 10^9$  conidia  $g^{-1}$  in tests 1 and 2, respectively) and corn ( $0.6 \times 10^9$  conidia  $g^{-1}$ ) appeared to be the least effective substrates for sporulation.

TABLE 2. Effect of moisture content on wheat seed colonization by *A. flavus* strain AF36 incubated for 24 h

Moisture content (%)	Colonization visual assessment scale 1-5*	
	Upper surface	Lower surface
Control†	1.0 a	1.8 a
10	1.0 a	1.5 a
12	1.0 a	2.1 a
15	1.0 a	2.0 a
20	1.0 a	2.0 a
25	4.8 b	4.8 b
30	4.8 b	4.8 b
35	4.8 b	4.8 b

\*Numbers in a column followed by a common letter are not significantly different using Tukey's HSD test. Fungal colonizations of the dorsal (upper) and ventral or crease (lower) surfaces were scored separately using a 1-5 scale where 1 = no colonization visible; 2 = 1-5 mycelial strands visible; 3 = 6-20 mycelial strands visible; 4 = 21-50 mycelial strands visible and 5 = > 50 mycelial strands visible.

†Control not inoculated.

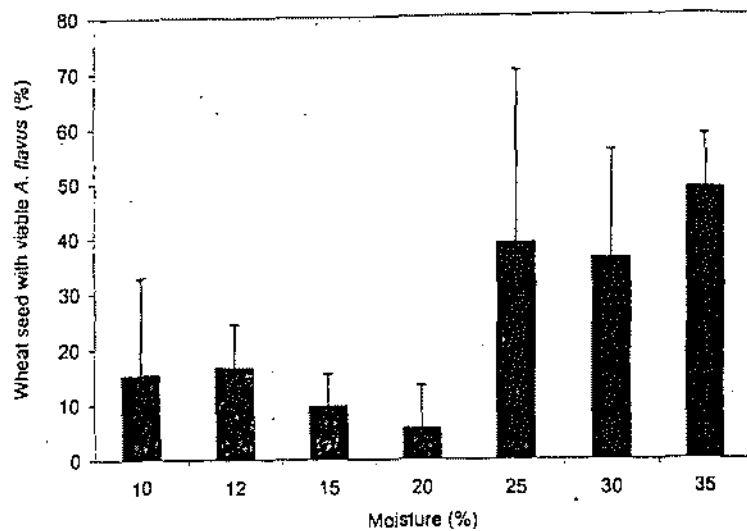


FIGURE 4. Effect of disinfection with 70% ethanol for 20 min on survival of *A. flavus* strain AF36 in wheat incubated at different moisture contents. Standard deviations (SD) of the means are indicated.

TABLE 3. Effect of substrate on the quantity of conidia produced by *A. flavus* strain AF36 during 7 days incubation.

Test 1		Test 2		Substrate	Hundred grain wt g, $\pm$ SD	Conidia/g
Substrate	Conidia*	Substrate	Conidia			
Wheat	11.4 a	Oats	9.2 a	Oats	2.9 (0.12)	36
Rye	11.6 ab	Wheat	7.2 ab	Wheat	2.9 (0.07)	36
Rice	9.3 ab	Rice	6.3 abc	Rice	1.5 (0.01)	67
Oats	7.0 ab	Finger millet	6.8 abc	Finger millet	1.0 (0.02)	176
Finger millet	6.5 bc	Soybeans	4.6 bcd	Sorghum	2.6 (0.13)	38
Soybeans	3.8 cd	Soybeans	3.8 bcd	Soybeans	2.0 (1.25)	5
Black-eyed peas	3.3 d	Rye	3.3 cde	Rye	3.5 (0.10)	23
Black beans	2.7 d	Black-eyed peas	3.3 cde	Black-eyed peas	22.6 (0.13)	4
Pima cottonseed	0.8 e	Black beans	3.2 cde	Black beans	19.3 (0.76)	5
		Barley	3.2 de	Barley	4.7 (0.04)	21
		Pima cottonseed	1.7 ef	Pima cottonseed	12.4 (0.68)	6
		Corn	0.6 f	Corn	36.0 (0.34)	3

\*Conidia  $g^{-1} \times 10^4$ .\*Means separation based on square root transformed data using Tukey's HSD test at  $P = 0.05$ . Numbers followed by the same letter are not significantly different.

Not all substrates were consistent between the two test runs. For example, rye produced  $11.6$  and  $3.5 \times 10^4$  conidia  $g^{-1}$  in tests 1 and 2, respectively. In addition, seed size varied greatly (100 grain weights ranged from  $0.6$  g for finger millet to  $36$  g for corn). There was a consistent negative correlation between sporulation capacity of the inoculated substrate and seed weight (in test 1,  $-0.7360$ ,  $P = 0.001$ , and in test 2,  $-0.6590$ ,  $P = 0.001$ ). Smaller seeds tended to produce greater numbers of spores/g of substrate.

#### Stability and Viability of the Wheat Seed/AF36 Formulation

*Effect of time.* Regression analysis ( $y = 0.005x - 10.08$ ,  $R^2 = 0.001$ ) showed there was no effect of storage period at room temperature ( $18-25^\circ\text{C}$ ) on spore yield of the biopesticide over a 29-month period from 12 May 1995–15 October 1997 (Figure 5).

*Effect of moisture content (seed drying periods).* In both tests, moisture content as high as 37% (w/w) in sealed containers did not influence viability over an 8-month period at room temperature ( $18-25^\circ\text{C}$ ). The colonized seed remained full capacity to produce conidia at all moisture contents (3–37% w/w). However, the fungus grew with atypical, fluffy white growth from a few of the seed stored at 26–30% (w/w) moisture. The fungus grew during storage at moistures above 15% (w/w). This growth resulted in undesirable clumping of the product. The moisture content of the colonized wheat rapidly fell in the drying oven from ca 35% (w/w) to ca 8% (w/w) over 48 h (Figure 6). Wheat moisture was reduced to below 15% (w/w) within 24 h.

*Effect of heat.* Extreme heat killed *A. flavus* (Table 4). When seed was heated for 20 min (Figure 7), viability decreased between  $70$  and  $90^\circ\text{C}$  ( $r = -0.7508$ ,  $P = 0.001$ , for tests 1 and 2 combined). At  $80^\circ\text{C}$ , viability of AF36 on wheat declined rapidly ( $r = -0.6377$ ,  $P = 0.05$ , for tests 1 and 2 combined), with a 50% loss of viability within 4 min and 90% loss after 40 min. Typical temperatures of exposure that the biocontrol agent was subjected to during shipping and on farm storage in western Arizona are shown in Figure 8. Maxima did not exceed  $58.6^\circ\text{C}$ .

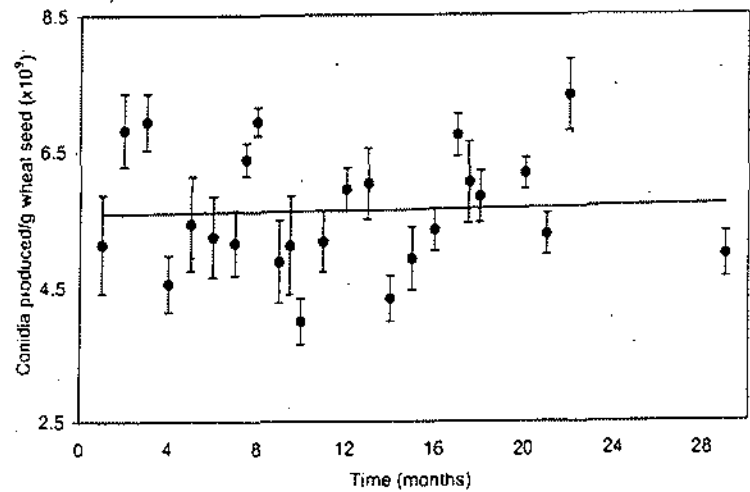


FIGURE 5. Viability of wheat seed inoculated with *Aspergillus flavus* strain AF36 at room temperature (18–25°C) during a 29 months period. The regression solution is linear,  $y = 0.005x + 0.048$ ,  $R^2 = 0.001$ .

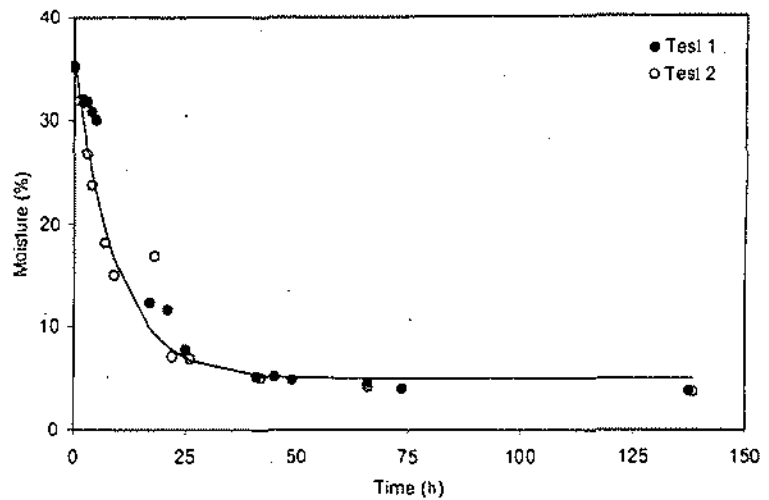
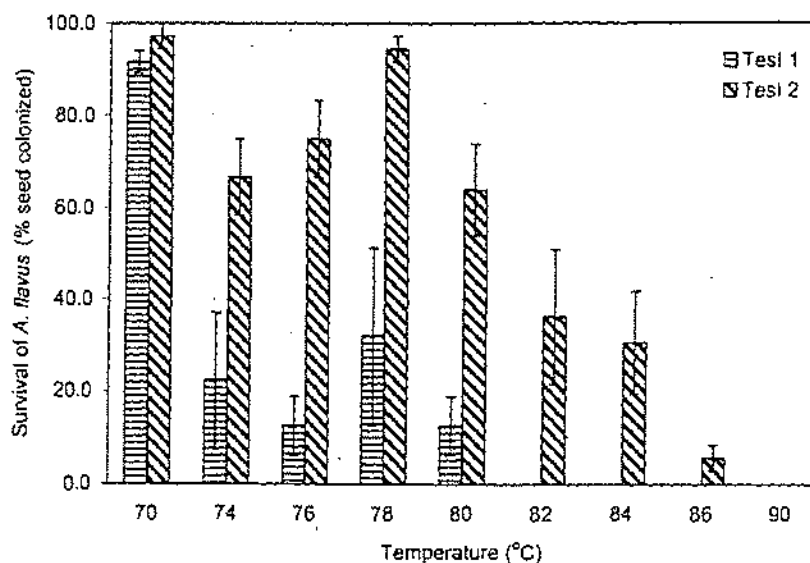


FIGURE 6. Influence of drying at 58°C on moisture content of wheat seed colonized by *A. flavus* strain AF36. The regression solution is an asymptotic model,  $y = 10.65 - 1 - 0.1(2e^{-0.11x})$ ,  $R^2 = 0.95$ .

TABLE 4. Effect of temperature on survival of *A. flavus* strain AF3b on colonized wheat seed

Temperature (°C) <sup>a</sup>	Survival (%) <sup>b</sup>	
	Test 1	Test 2
Control	100 a	100 a
60	100 a	100 a
70	100 a	95.8 a
80	33.3 b	19.4 b
90	0 c	0 c
100	0 c	0 c
110	0 c	0 c

<sup>a</sup>Temperature maintained for 20 min.<sup>b</sup>Numbers are means of three replicates. Values followed by a common letter are not significantly different using Tukey's HSD test.FIGURE 7. Effect of temperature on viability of *A. flavus* strain AF3b on wheat seed. Standard errors of the means are indicated.

## DISCUSSION

For a biopesticide to be useful it must meet specific criteria: it must have sufficient shelf life both during culture and in the release environment, have adequate efficacy, minimal production costs, and good shelf life, preferably at least 18 months (Couch & Ignoffo, 1981; Cotty *et al.*, 1994). Efficacy of wheat seed colonized by *A. flavus* in displacing aflatoxin-producing strains and reducing aflatoxin contamination of cottonseed has been demonstrated in field plot experiments (Cotty, 1994), and pilot studies in commercial settings (Raymer, 1995). The current study highlights some of the advantages of the wheat seed formulation compared to potential alternatives already considered (Daigle & Cotty, 1995, 1997). The wheat seed/*A. flavus* product is particularly straightforward to produce and eliminates

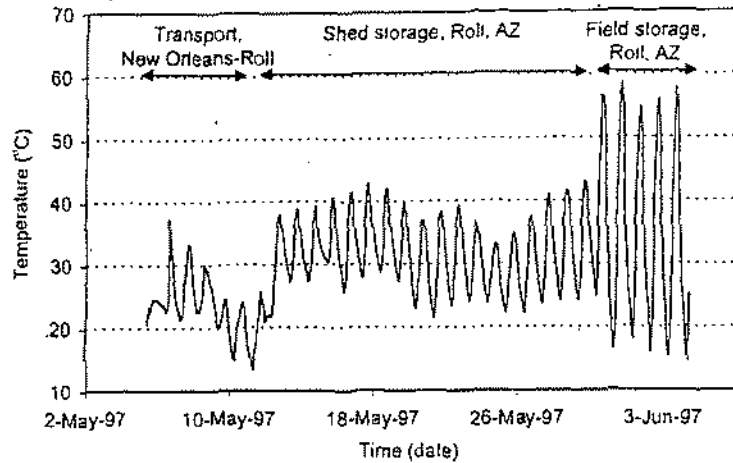


FIGURE 8. Temperatures experienced by wheat seed colonized by *A. flavus* strain AF20 during commercial use in western Arizona. The material was manufactured in New Orleans, Louisiana, and shipped to Roll, Arizona, where it was stored on farm until use.

cumbersome manufacturing needs required in alginate pellet formulation (Daigle & Cotty, 1997).

Using a natural substrate like wheat seed is practical. Wheat seed is relatively inexpensive (ca. US\$0.18–0.26 kg<sup>-1</sup>), readily available and already has characteristics that are ideal for application (granular and flowable). Wheat seed also has tough outer coats that prevent damage to the dried product. Colonization of the wheat prior to application reduces the likelihood that the wheat will be exploited by other, unintended microorganisms. The product has been successfully applied in commercial operations at 10 kg ha<sup>-1</sup> using a granular applicator (for example, a Gandy Box), a muck spreader and an aerial applicator. The latter will be particularly important if large areas must be treated over relatively short periods.

*A. flavus* grows extremely well on wheat, and produces excellent spore yields ( $5.7 \times 10^9$ – $7.9 \times 10^9$ ) after 7 days incubation. Previous studies with artificial media (Daigle & Cotty, 1995, 1997) indicated that alginate formulations produce more conidia than wheat seed ( $4.0 \times 10^9$  g<sup>-1</sup> vs  $1.0 \times 10^9$  g<sup>-1</sup>, respectively after 7 days incubation). However, the advantage in conidial production may not warrant the additional cost of alginate production (total bulk costs up to US\$5.76 kg<sup>-1</sup>; Daigle & Cotty, 1997). Although as simplified mass production procedures are developed, the cost for alginate may fall considerably (Daigle *et al.*, 1997; Daigle *et al.*, 1998). A further potential advantage of wheat over alginate that has not been tested may be the duration of its integrity under field conditions, thereby prolonging spore production. In the current study, wheat was consistently an excellent substrate for spore production. Substrates with larger particle sizes produced fewer spores. This may be explained by the surface area to volume ratios of the different seeds. Large seeds have a proportionately smaller surface area from which to produce conidia. In the current test, seed size variation may have interfered with detection of influences from nutritional differences among substrates. To evaluate these influences, the various substrates should probably have been ground, and uniformly pelletized.

Sterile wheat colonized by *A. flavus* shows remarkable long term stability and tolerance of heat and moisture. In these studies the fungus survived for 29 months with no loss in viability when stored at room temperature (and ca 6% moisture). Even at 35% (w/w) moisture, survival extended throughout the 8 month study.

At 6% (w/w) moisture, the end use product survived 70°C for at least 20 min. Indeed, the product is routinely dried during manufacture at 58°C for 48 h and retains 100% viability. Previous studies (Daigle & Cotty, 1995) using alginate formulations indicated temperature in excess of 32°C and RH in excess of 50% resulted in reduced viability within a few months. The extensive product stability observed in the current study makes production, transport, and use more commercially compatible. This high stability should also allow survival during unfavorable periods in the field after application. Air temperatures in the field in Arizona frequently exceed 40°C (AZMET data, Roll, Arizona, July–September on the world wide web at <http://www.az.arizona.edu/azmet>; Bock, unpublished data), but maxima exceed 50°C only occasionally. However, after application wheat may be exposed in full sun on the soil surface to temperatures over 70°C (Bock, unpublished data). If applications are made immediately prior to irrigation, prolonged exposure to temperatures in excess of 60°C will be avoided. It might be prudent to delay applications until canopy shade can ameliorate the temperature to which the product is exposed.

Cost saving may be made at several stages in the manufacturing process. Colonization can occur with minimal water (25% moisture) and reduced incubation (18 h). In the current study drying to 15% was sufficient to prevent fungal growth in storage. This is considerably less drying than previously employed. If fungal growth occurs before application flowability and dispersability will be reduced. Furthermore, premature fungal growth wastes resources needed after application.

The amount of colonized wheat seed added in the field is relatively low (10 kg ha<sup>-1</sup>). This application rate effectively reduces aflatoxin without increasing the overall quantity of *A. flavus* on the crop (Cotty, 1994). The production of colonized wheat, as outlined here, requires relatively small quantities of conidia. To put the required quantity in perspective, consider growing *A. flavus* on wheat seed and using the conidia from that seed for the bulk inoculation of further seed. Assuming 1 g (about eight grains) of wheat seed produces  $7.0 \times 10^9$  conidia, and each kg of seed requires  $10^9$  conidia for adequate colonization, it should be possible to produce 1000 kg of final product (a quantity sufficient to treat 100 ha!), with the conidia produced from just 1 g of colonized sterile wheat. In addition, the *A. flavus* wheat seed formulation is axenic, while the alginate system suffers microbial contamination during the production cycle (Daigle *et al.*, 1998).

Wheat seed colonized with toxigenic *A. flavus* may have application on many crops where aflatoxin contamination is a recurrent problem (Brown *et al.*, 1991; Dorner *et al.*, 1992). Similar formulations may also be relevant to aflatoxin control in less developed countries where aflatoxin can be a serious problem on staple food like corn (Sematou *et al.*, 1997) and groundnuts (McDonald & Mchan, 1989). It seems likely that specific strains well adapted to, and native in target regions and crops will be needed. However, the manufacturing procedures outlined here should be widely adaptable even in relatively low technology areas.

Finally, grain and other natural products are routinely used as media for culture of diverse fungi (CABI, 1983). This may indicate that similar, inexpensive production methods may be applicable to formulation of other biopesticides. For example, *Trichoderma* sp. is effectively applied in a pregelatinized starch/floor granular formulation (Lewis *et al.*, 1995), but work in India (Sarwant *et al.*, 1995) indicates *Trichoderma* sp. grown on agricultural waste also controls fungal pathogens.

Ultimate commercialization of the wheat seed *A. flavus* formulation and manufacturing process will depend on economics of production versus the benefits of aflatoxin control. It seems likely that haring capital investment, the costs of producing sterile wheat colonized by an toxigenic strain of *A. flavus* will be low. The ultimate success of such a venture may

lie with the perception of the economic benefits and risks both within agricultural communities and within society as a whole.

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# 14 Genetic and Biological Control of Aflatoxigenic Fungi

*D. Bhatnagar, P.J. Cotty, and T.E. Cleveland*

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## I. INTRODUCTION

Aflatoxins, metabolites produced by several members of *Aspergillus* section *Flavi*, particularly, the fungi *Aspergillus flavus* and *A. parasiticus*, occur in food and feed crops before harvest and during storage. These compounds are toxic and extremely carcinogenic when introduced into animal systems; therefore, many investigations have focused upon identifying technologies to inhibit fungal growth and/or aflatoxin synthesis in crops to prevent contamination and subsequent consumption of these toxic compounds by animals and humans.

A "gene cluster" (over 70 kilobases in size) in *A. flavus* and *A. parasiticus* has recently been identified upon which reside almost all the genes involved in aflatoxin biosynthesis. A few of these genes appear to be "environmentally responsive," and their expression may be regulated by several factors that affect aflatoxin production, such as temperature, pH, plant metabolites and carbon, and nitrogen sources. The molecular regulation of this gene cluster has been characterized in some detail, including the identification of a regulatory gene and a common regulatory relationship between fungal development and toxin synthesis. This critical information on aflatoxin biosynthesis provides us with an opportunity to target specific sites to interfere with aflatoxin formation in plants.

*Aspergillus flavus* is the most common causal agent of aflatoxin contamination. Communities of fungi that fall within the species *Aspergillus flavus* are highly diverse. Strains of *A. flavus* may belong to different genetically isolated groups that have widely varying abilities to produce aflatoxins. Some natural *A. flavus* strains produce no aflatoxins. These atoxigenic strains have been "seeded" into agricultural fields so that the atoxigenic strain predominates the fungal communities. The increase in atoxigenic strain occurrence results in reduced vulnerability of crops to contamination without an increase in the overall quantity of *A. flavus* in the field. Identification of critical genes governing aflatoxin formation could also lead to the conversion of any ecologically successful *A. flavus* or *A. parasiticus* strain, through gene disruption, into a designer non-aflatoxigenic biocontrol strain that could be used in aflatoxin management programs directed at competitively excluding aflatoxigenic strains in specific environments.

Efforts are also underway to inhibit fungal growth and/or aflatoxin formation through enhancement of host-plant resistance. A great deal of knowledge about the mechanisms involved in natural resistance in certain corn inbreds has been produced through inoculation of kernels with reporter gene-containing *A. flavus* (tester strains). By monitoring the degree of fungal infection and toxin production, resistance mechanisms have been identified on kernel surfaces, beneath the kernel pericarp, and in the embryo. Certain kernel physical components and antifungal proteins have also been shown to correlate with high levels of resistance in corn varieties. Additional studies have led to the identification of a variety of antifungal (to *A. flavus* infection) peptides, proteins, and genes from several host and non-host species. Knowledge gained from studies that establish plant-*Aspergillus* interactions, host resistance mechanisms, and consequent aflatoxin formation are being used in plant breeding and genetic engineering strategies to suppress aflatoxin production in crops.

## II. AFLATOXIN-PRODUCING FUNGI

All aflatoxin-producing fungi may be assigned taxonomically to *Aspergillus* section *Flavi*. These include *Aspergillus flavus* and *Aspergillus parasiticus*, as well as several less common taxa including *Aspergillus nomius*.<sup>1</sup> The aflatoxin-producing group is considerably more complex than previously thought; for example, strains of *Aspergillus tamaritii* have recently been shown to produce aflatoxins,<sup>2</sup> and new taxa may soon be described as important aflatoxin producers, historically only found in the previously named taxa.<sup>3-4</sup>

*A. flavus*, a species that is ubiquitous in warm tropical and desert environments,<sup>1,5</sup> is a highly diverse asexual species that can be divided on the basis of physiological, morphological, and genetic criteria; however, most crop contamination with aflatoxins is apparently caused by either the S or the L strains of *A. flavus*.<sup>6</sup> The S strain can be separated on the basis of sclerotial morphology and habitat. The S-strain isolates produce, on average, significantly higher levels of aflatoxins than typical or L-strain isolates. In some agricultural regions, S-strain isolates dominate and are responsible for most of the aflatoxin-producing potential of the resident *A. flavus* communities.<sup>7-9</sup> However, S-strain isolates frequently vary in virulence, with certain isolates failing to produce the primary pectinase needed to ramify through host tissues.<sup>10,11</sup> Thus, isolates with the greatest aflatoxin-producing potential do not always have high virulence. Conversely, isolates that do not produce aflatoxins in crops may be very effective in colonizing and ramifying through plant host tissue.<sup>6</sup>

*Aspergillus flavus* populations within agricultural fields are complex, with members of the population further divided genetically by vegetative compatibility which limits gene flow between dissimilar individuals.<sup>12</sup> Both S-strain and L-strain isolates are composed of many vegetative compatibility groups (VCGs).<sup>13,14</sup> Genetically distinct *A. flavus* strains frequently interact during dispersal, growth, and crop infection.<sup>1,13</sup> Multiple VCGs commonly reside within a gram of soil or infect a single seed.<sup>13</sup> The influence of these individuals on each other during crop infection may vary widely.<sup>15</sup> VCGs evolve largely when distinct clonal lineages gradually diverge as they spread spatially. This gradual divergence among VCGs can be measured by distinct random amplified polymorphic DNA (RAPD) markers and isozyme profiles.<sup>16</sup> VCGs may also differ both physiologically and morphologically,<sup>14,17</sup> and the characteristic most frequently examined is the aflatoxin-producing ability.

The aflatoxin-producing ability of *Aspergillus flavus* isolates varies widely, as well. A significant proportion of *A. flavus* populations may not produce aflatoxins (atoxigenic),<sup>7</sup> whereas others have the potential to contaminate infected seed with over a million parts per billion (ppb). Isolates within a VCG tend to have similar aflatoxin-producing potentials, and certain VCGs have no known members that produce aflatoxins.<sup>14,17</sup> The lack of aflatoxin-producing ability has been most thoroughly studied in isolates of *A. flavus* and *A. parasiticus* and other aspergilli used in food production.<sup>18</sup> These latter isolates, typically named *A. oryzae* and *A. sojae*, are domesticated strains of *A. flavus* and *A. parasiticus*, respectively, and have been used to produce soy sauce and other fermented foods for centuries.<sup>4,19</sup>

### III. ECONOMIC SIGNIFICANCE OF AFLATOXIN CONTAMINATION

Aflatoxins have been found in many foods of animal and plant origin, including corn meal, peanuts, cottonseed, spices, cassava, pistachio nuts, rice, cocoa, bread, macaroni, copra, Brazil nuts, oilseeds, pumpkin seeds, meat pies, milk, cheese, sausage, and cooked meat. In the U.S., frequent preharvest contamination of corn, cotton, peanuts, and tree nuts is of major concern because of the economic impact of destroying contaminated crops.

Since their discovery in 1960, aflatoxins have been implicated in carcinogenicity, mutagenicity, teratogenicity, hepatotoxicity, and aflatoxicosis. Currently, 18 different aflatoxins are known. The most important members of this family of toxins are B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, G<sub>2</sub> (see Figure 14.11), M<sub>1</sub>, and M<sub>2</sub>. Of these, aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) is the most common and also the most carcinogenic. AFM<sub>1</sub> is a contaminant in the milk of cows fed with AFB<sub>1</sub>-contaminated feed and is considerably less toxic than AFB<sub>1</sub>.

Epidemiological studies have provided evidence of the carcinogenicity of aflatoxin B<sub>1</sub> to humans (for several reviews, see reference 20). The liver is the primary target organ in many animal species; however, tumors in other organs have also been observed in aflatoxin-treated species. Ironically, this effect arises as a result of the detoxification response in animals. Highly reactive aflatoxin derivatives (8,9-epoxy-aflatoxin B<sub>1</sub>) can intercalate DNA and form DNA-aflatoxin adducts. Subsequent cellular repair of the adducts often leads to G-to-T transversion in the coding region of genes, particularly that of the tumor suppressor gene, p53. A very high incidence (67%) of liver carcinomas in Senegal, China, Swaziland, and Mozambique bear the characteristics of aflatoxin-induced mutation of the p53 tumor suppressor gene. This mutation has also been associated with liver cancer in Mexico. The binding of AFB<sub>1</sub> to DNA also leads to the formation of single-stranded gaps. As a result, it inhibits DNA polymerase activity at DNA binding sites. This stimulates an error-prone repair system that may induce mutation. Furthermore, it has been suggested that AFB<sub>1</sub> is teratogenic due to its prenatal effects on certain animals. Its inhibitory effect on protein synthesis of eukaryotic cells can impair differentiation in sensitive primordial cells. According to epidemiological studies, raising the permissible limits of aflatoxins in foods in the U.S. would not greatly increase the incidence of liver cancer; however, there is a worldwide epidemic of hepatitis C, and hepatitis C patients with impaired liver function may be much more susceptible to aflatoxins than healthy people.

The national economy would be affected adversely both by the losses incurred by crop and livestock producers when aflatoxin-contaminated crops are destroyed due to regulatory restrictions and by the multiplier effect this would have on other industries as a result of the reduced spending power of producers.<sup>21,22</sup> Additionally, the costs of chemical analyses, quality control and regulatory programs, research and development, extension services, law suits, and human illness must all be borne by the national economy. The direct cost of aflatoxin contamination in corn in 1980 to all of the southeastern states was estimated to be greater than \$237 million.<sup>23</sup>

While the short-run costs are substantial for the individual and for society, they may be greater in the long run if recurrent aflatoxin (for example, in corn) cannot

be eliminated or detoxified. Farmers who are unable to market their corn will ultimately shift acreage to other crops such as soybeans or grain sorghum, which have less year-to-year risks and considerably less net returns. Thus, the growers in affected areas will have fewer cropping options and be forced to bear market forces on the few crops they can grow.

The economics of aflatoxin contamination are an issue primarily relevant to developed countries where food is in ample supply. In developing countries, where food is sometimes in short supply, long-term health implications of aflatoxin contamination are commonly overlooked. However, economies of developing countries could be seriously affected when the presence of even the smallest amount of toxin in export commodities is rejected by countries that strictly adhere to regulatory guidelines for levels of toxin in agricultural products for human or animal consumption.

#### IV. CONTROL OF PREHARVEST AFLATOXIN CONTAMINATION

Aflatoxin formation before or after crop harvest cannot be prevented thus far, but it can be reduced by appropriate management practices. Due to the human and animal health implications, intense efforts worldwide are underway to remove aflatoxin from food and feed supplies. Attention has been focused on the preharvest control of aflatoxin contamination, because that is when the fungi first colonize host tissues. This emphasis would obviate the need to detoxify large quantities of contaminated materials and avoid the uncertainties of gaining approval from regulatory agencies for the use of detoxified seeds for animal feed or human food. However, control strategies should also include methods for detoxifying contaminated products resulting from prevention measures that are not always completely successful. Detoxification can prevent total loss of valuable foodstuffs and reduce the burden of contamination at the farm gate (for reviews on detoxification procedures see references 20, 24-26).

##### A. CONVENTIONAL METHODS

Several agronomic practices have been shown to reduce preharvest aflatoxin contamination in certain crops,<sup>27</sup> including the use of pesticides (fungicides and insecticides), altered cultural practices (such as irrigation), and the use of resistant varieties. However, such procedures have only a limited potential for reducing aflatoxin levels in the field, especially in years when environmental conditions are particularly favorable to the contamination process (for review, see reference 28).

##### B. USE OF NATURAL PRODUCT INHIBITORS TO CONTROL AFLATOXIN CONTAMINATION

There are several plant-derived inhibitors of aflatoxin synthesis, and this subject has been reviewed extensively.<sup>29</sup> Inhibitors with unknown modes of action have been discovered in our laboratory<sup>30,31</sup> that could be directly applied to crops in the field. Examples of natural products that may have potential in augmenting host-plant

resistance against *A. flavus* infection are certain plant-derived volatile compounds.<sup>30,32-34</sup>

### C. CONTROL OF AFLATOXIN CONTAMINATION THROUGH BIOTECHNOLOGY

Because conventional methods are only partially effective and are not expected to achieve the extremely low or negligible levels of aflatoxin required to meet regulatory guidelines for the sale and export of commercial food and feed, there is an increasing need to develop new technology to reduce and eventually to eliminate preharvest aflatoxin contamination. Three biotechnological approaches are being developed to exclude toxigenic fungi from their environmental niches and to regulate fungal growth or aflatoxin biosynthesis in crops:

1. Inhibit biosynthetic or secretory processes responsible for aflatoxin accumulation.
2. Replace aflatoxigenic strains with non-aflatoxigenic (biocompetitive) strains in the field.
3. Enhance host resistance by marker-assisted plant breeding or by genetic engineering of plant varieties to specifically express antifungal agents in the susceptible plant tissues (e.g., infected seed tissues).

## V. AFLATOXIN BIOSYNTHETIC PATHWAY

Elimination of preharvest aflatoxin contamination through plant-induced inhibition of biosynthetic or secretory processes responsible for toxin production<sup>35-37</sup> would significantly benefit from additional knowledge about the fundamental molecular and biological mechanisms that regulate the synthesis of aflatoxin by the fungus. Previous studies have determined that aflatoxins are synthesized by the polyketide metabolic pathway (for reviews, see references 38-40). The generally accepted scheme for aflatoxin biosynthesis is acetate → polyketide precursor → norsolorinic acid, NOR → averantin, AVN → 5'-hydroxyaverantin, HAVN → averufanin, AVNN → averufin, AVF → versiconal hemiacetal acetate, VHA → versiconal, VAL → versicolorin B, VERB → versicolorin A, VERA → demethylsterigmatocystin, DMST → sterigmatocystin, ST → O-methylsterigmatocystin, OMST → aflatoxin B<sub>1</sub>, AFB<sub>1</sub> (Figure 14.1).

Specific enzyme activities, including those of reductase, dehydrogenase, cyclase, desaturase, P-450 monooxygenase, and O-methyltransferase, have been associated with precursor conversions in the aflatoxin pathway (for reviews, see references 38-41). Some of these enzymes have been partially purified, whereas others have been purified to homogeneity (for reviews, see references 38-41 and references therein). The genes encoding most of these enzymes have been cloned (for reviews, see references 36, 37, 42) (Figure 14.1). Alternate pathways may exist at several steps in the aflatoxin pathway;<sup>43</sup> therefore, more than one enzyme may catalyze the same reaction — for example, the reductase/dehydrogenase encoded by *nor1*, *norA*, and *norB*.<sup>44,45</sup> Also, independent reactions and different chemical precursors involved

in AFB<sub>1</sub> and AFB<sub>2</sub> syntheses are catalyzed by common enzyme systems — that is, *O*-methyltransferases encoded by *omtA* and *omtB*<sup>43,46-50</sup> and the P-450 oxidoreductase encoded by *ordA*.<sup>51,52</sup>

Genetic studies of *A. flavus* and *A. parasiticus* were hampered by the lack of a sexual stage in these fungal species. Nonetheless, by means of parasexual cycle analysis, over 30 genes have been mapped to eight linkage groups.<sup>53,54</sup> Pulsed-field gel electrophoresis has helped resolve karyotypes and define genetic maps of these imperfect fungi.<sup>55,56</sup> Karyotyping of several *A. flavus* and *A. parasiticus* strains shows that there are six to eight chromosomes ranging in size from approximately 3 to 27 Mb.<sup>53</sup> The aflatoxin genes were mapped to linkage group VII in *A. flavus*.<sup>56</sup>

Genetic complementation has been a valuable tool in the cloning of aflatoxin biosynthesis genes.<sup>57</sup> Chromosomal walking and cross-hybridization studies have established that genes for aflatoxin synthesis in *A. parasiticus* and *A. flavus*,<sup>58-60</sup> as well as sterigmatocystin synthesis in *A. nidulans*, are clustered.<sup>61</sup> One of these genes, *aflR*, involved in the transcriptional regulation of aflatoxin and sterigmatocystin biosynthesis,<sup>62-64</sup> has been characterized from *A. parasiticus*, *A. flavus*, and *A. nidulans*. The function of AFLR proteins is conserved among the three aspergilli.<sup>64</sup> Expression of genes in the AF/ST clusters is co-regulated by AFLR, and AFLR binds to the promoters of aflatoxin biosynthesis genes.<sup>65-67</sup> AFLR also appears to regulate its own expression.<sup>62</sup> The transcription activation domain in *A. parasiticus* AFLR has been localized to its carboxy-terminal region.<sup>68</sup> Recently, a regulatory association between aflatoxin biosynthesis and fungal development has also been suggested.<sup>69-71</sup> Regulatory factors other than AFLR that play a role in aflatoxin gene expression as well as in fungal survival must be identified to provide effective manipulation of these toxigenic fungi.

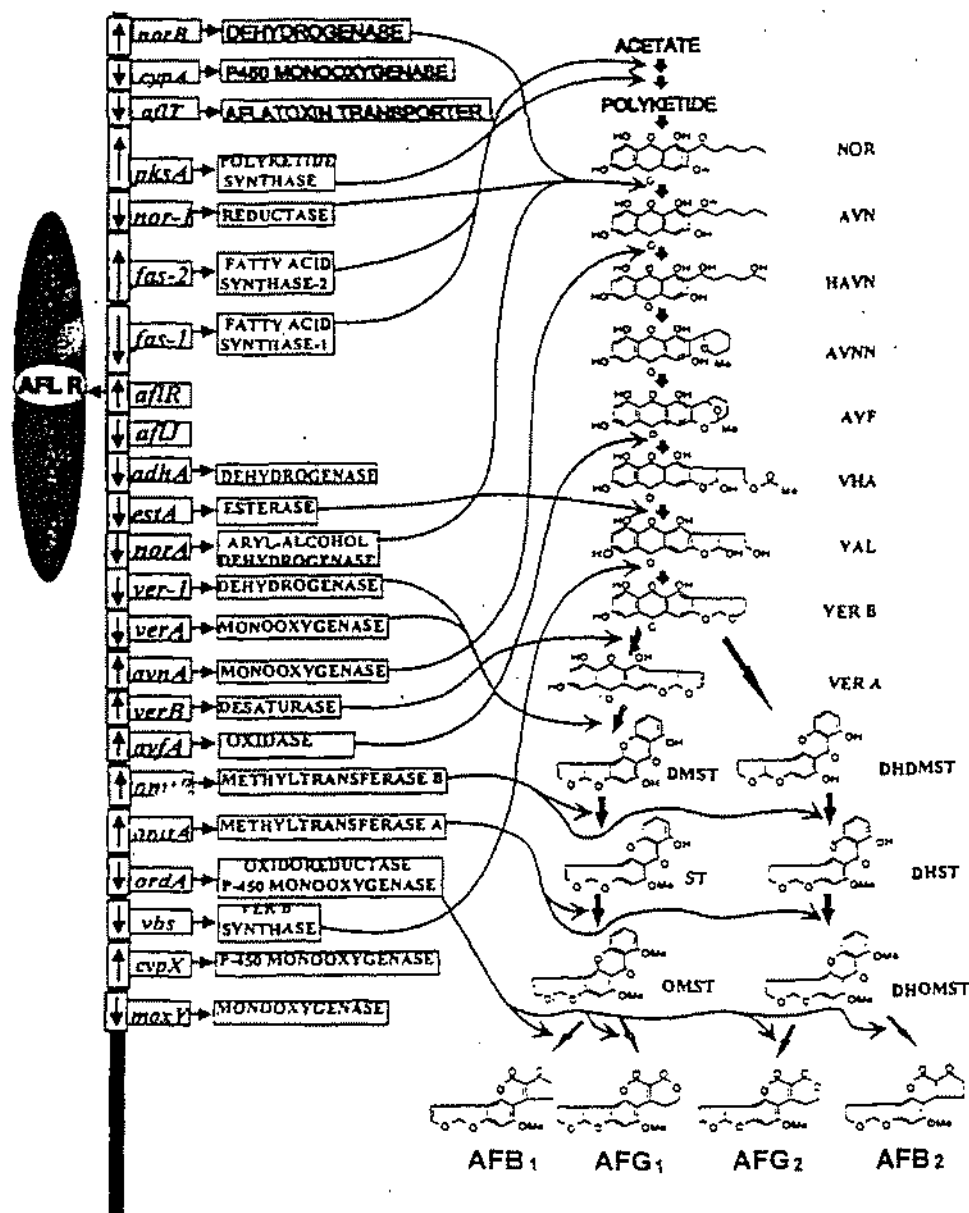
Expression "probes" based on cloned aflatoxin genes and the use of reporter gene technology could be used to identify phytological agents that naturally inhibit aflatoxin biosynthesis.<sup>72-74</sup> This knowledge could then be used to improve resistance to aflatoxin contamination through marker-assisted plant breeding or genetic engineering procedures. Plant chemicals from crops vulnerable to aflatoxin that modulate aflatoxin biosynthesis have been reported;<sup>30,33,34,75-77</sup> however, additional knowledge is needed with regard to the identity, synthesis, and tissue sites of these plant components that influence aflatoxin synthesis during plant-fungus interaction.

The complete characterization of aflatoxin genes and their regulation not only has been extremely beneficial in our understanding of how the toxin is produced by the fungus when it invades a crop, but has also aided in the success of other projects seeking to develop non-aflatoxigenic biocompetitive fungi or to monitor crop resistance to fungal growth and aflatoxin formation.

## VI. STRAIN INTERACTIONS AND BIOLOGICAL CONTROL

Not only may individual isolates, strains, and VCGs differ in aflatoxin-producing potential, but communities of fungi found in different areas may also have different aflatoxin-producing potentials.<sup>7,8</sup> The latter may contribute to variability in crop vulnerability to aflatoxin contamination in different regions and different fields. The lack of correlation between the ability to produce aflatoxins and a producing strain's

ability to colonize and infect developing cotton bolls<sup>6</sup> suggested that atoxigenic strains of *A. flavus* could exclude aflatoxin-producing strains through competition during infection of developing crops and thereby prevent aflatoxin contamination.<sup>6,7,78,79</sup> In both greenhouse and field experiments, simultaneous wound inoculation of developing cotton bolls and corn ears with toxigenic and atoxigenic strains resulted in reductions in aflatoxin contamination of the developing crop parts as compared with controls inoculated with only the toxigenic strains.<sup>80,81</sup> Although not all atoxigenic strains reduce contamination by aflatoxin-producing strains during co-



infection of crops, certain atoxigenic strains consistently cause reductions of 90% or more.<sup>15,81</sup>

Greenhouse and laboratory tests indicate that the mode of action of the atoxigenic strains is primarily through competitive exclusion.<sup>82</sup> Application of atoxigenic strains in the field has also been shown to be effective at preventing postharvest aflatoxin contamination both when the crop is infected by aflatoxin producers naturally in the field and when inoculated after harvest.<sup>80</sup> Taken together, the greenhouse and laboratory tests suggest that competitive exclusion of aflatoxin-producing strains of *A. flavus* with atoxigenic strains of the same fungal species may provide an efficient method for reducing aflatoxin accumulation throughout crop production, storage, and utilization.<sup>6,78,81,83</sup> These tests, however, rely on direct application of the atoxigenic strains to either fresh wounds in the crop or to harvested kernels. To circumvent this drawback, Cotty et al.<sup>84</sup> have developed a practical method for applying the atoxigenic strains in an agricultural setting. Efforts to use atoxigenic strains to prevent contamination is limited to three crops: peanuts and corn in southeastern

**FIGURE 14.1** Summary of the cluster of aflatoxin pathway genes, corresponding biosynthetic enzymes, and precursor intermediates involved in the aflatoxin B<sub>1</sub> and B<sub>2</sub> synthesis. The generally accepted aflatoxin B<sub>1</sub> and B<sub>2</sub> biosynthetic pathway in *A. parasiticus* and *A. flavus* and the identified enzymes for some specific conversion steps and cloned genes are schematically presented. The regulatory gene, *aflR*, coding for the pathway regulatory factor (AFLR protein), controls the expression of the structural genes at the transcriptional level. The *fas1*, *fas2*, and *pksA* gene products, fatty acid synthase, and polyketide synthase, respectively, are involved in the conversion steps between the initial acetate unit to the synthesis of the decaketide backbone in aflatoxin synthesis. The *nor1* gene encodes a reductase for the conversion of NOR to AVN. The *avnA* gene encodes a P-450 monooxygenase for the conversion of AVN to HAVN. The *aflJ* gene has also been demonstrated to be involved in the regulation of aflatoxin biosynthesis, but the role is under investigation. The *adhA* (homology to an alcohol dehydrogenase), *norA* (homology to an aryl-alcohol dehydrogenase), *ver1* (encoding a dehydrogenase), *avfA*, and *cyp450* gene products have been demonstrated to be functioning at various stages of the pathway, but their exact enzymatic role has not been fully characterized and is under investigation. The *omtA* gene encodes an *O*-methyltransferase for the conversion of ST to OMST and DHST to DHOMST. The *vbs* gene encodes a Ver B synthase (cyclase), which has been reported to be involved in the conversion of VHA to Ver B. The *ordA* gene encodes an oxidoreductase for the conversion of OMST to AFB<sub>1</sub> and DHOMST to AFB<sub>2</sub>, and is also involved in the conversion of OMST to AFG<sub>1</sub> and DHOMST to AFG<sub>2</sub>. The *estA* gene encodes an esterase involved in the conversion of VHA to VAL. The vertical bar on the left represents at least a 75-kb aflatoxin pathway gene cluster with identified genes shown in the open boxes. The names of the individual genes are labeled next to the open boxes. Arrows inside the open boxes indicate the direction of transcription. Arrows indicate the relationships from the genes to the enzymes they encode, from the enzymes to the bioconversion steps they are involved in, and from the intermediates to products in the aflatoxin bioconversion steps. Abbreviations: NOR, norsolorinic acid; AVN, averantin; HAVN, 5'-hydroxyaverantin; AVNN, averufanin; AVF, averufin; VHA, versiconal hemiacetal acetate; VAL, versiconal; Ver B, versicolorin B; Ver A, versicolorin A; DHST, dihydrosterigmatocystin; ST, sterigmatocystin; OMST, *O*-methylsterigmatocystin; DHOMST, dihydro-*O*-methylsterigmatocystin; AFB<sub>1</sub>, aflatoxin B<sub>1</sub>; AFB<sub>2</sub>, aflatoxin B<sub>2</sub>.

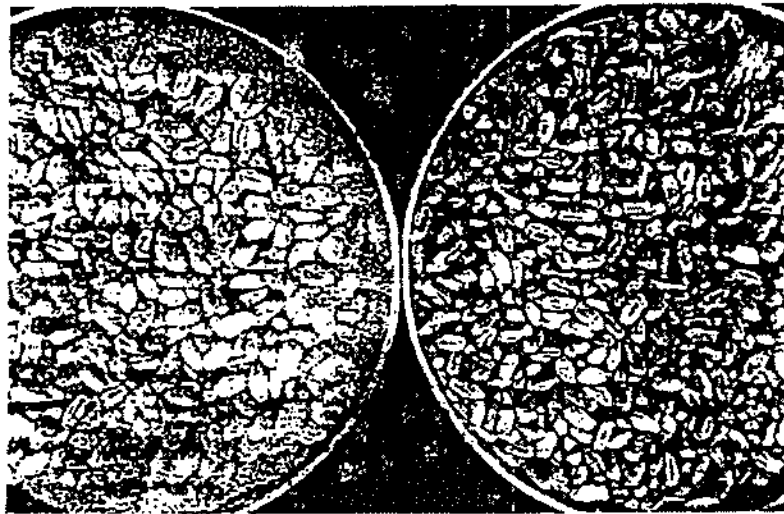


FIGURE 14.2 Cover of the 1998 Cotton Foundation annual report shows wheat colonized by an atoxigenic strain of *A. flavus* as it is applied (right) and after the fungus has grown out (left). The cotton industry has been enthusiastic about the potential for atoxigenic strain technology to prevent aflatoxin contamination.

U.S. and cottonseed in Arizona.<sup>80,83,85-88</sup> Emphasis at the Southern Regional Research Center has been on the application of atoxigenic strain technology to control aflatoxin contamination of cottonseed in regions of Arizona. In the U.S., aflatoxin contamination of cottonseed is most consistent and severe in the irrigated western desert.<sup>87</sup> Cottonseed produced in these valleys has a relatively high value per acre due to both high yields and high demand for cottonseed within the area.

#### A. DEVELOPING ATOXIGENIC STRAIN TECHNOLOGY

An aflatoxin-prevention technology based on atoxigenic strains of *A. flavus* was developed (Figure 14.2) for use in the region of Arizona with the most frequent and severe aflatoxin contamination of cottonseed.<sup>84</sup> For use in Arizona, a solid formulation of atoxigenic *A. flavus* has been developed in which whole wheat seeds are sterilized and colonized with the strain of choice.<sup>83,88</sup> This formulation has multiple year stability and tolerates exposures up to 70°C.<sup>88</sup> The fungus, in this formulation, can withstand both the severe conditions of on-farm storage during the summer months of the desert and direct exposure to Arizona's severe summer conditions after application.

For atoxigenic strains of *A. flavus* to be useful during crop production, they must be applied at a time and in a manner that allows them to compete successfully with aflatoxin-producing strains. In theory, application of an atoxigenic *A. flavus* strain when overall *A. flavus* levels are low should give the atoxigenic strain preferential exposure to the developing crop and thus the advantage in competing for crop resources during infection and during *A. flavus* population increases associated with crop production.<sup>1,87</sup> The atoxigenic strains are routinely applied at 10 lb per acre, but it should be emphasized that it is the timing of applications that dictates success.

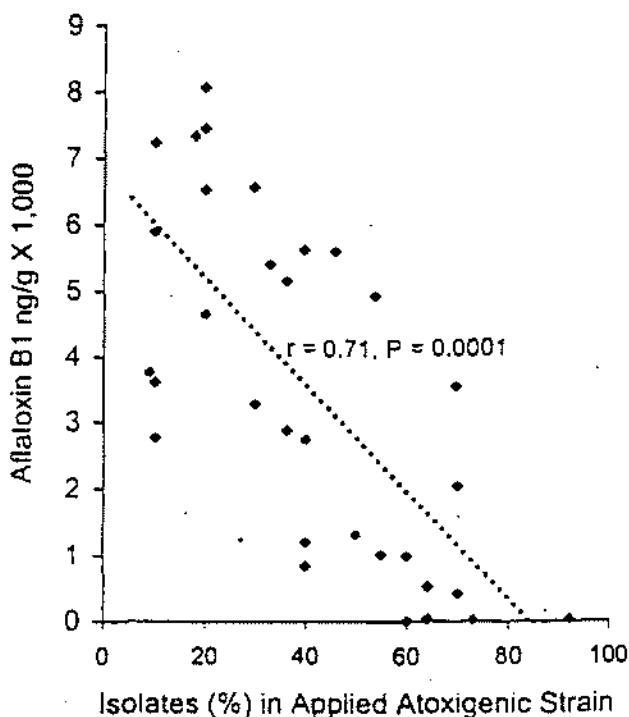


FIGURE 14.3 Relationship between the incidence of an applied atoxigenic strain in the infected portion of the crop and the quantity of aflatoxins in that crop. (Redrawn from Couty, P.J., *Phytopathology*, 84, 1270, 1994.)

During seasons when aflatoxin contamination is severe, *A. flavus* populations increase as the cotton crop is produced.<sup>89</sup>

Strains are seeded into cotton fields immediately prior to first bloom. As in greenhouse tests,<sup>15</sup> it was found that strains differ in efficacy in the field, and selection of the proper strain and the inoculum rate may potentially determine the extent of success of a biological control strain.<sup>90</sup> The strains are applied to the soil surface under the crop canopy in the form of colonized sterile wheat seeds. When the crop is subsequently irrigated, the atoxigenic strain utilizes the resources in the colonized wheat seed, sporulates, and disperses to the crop. Wheat seed colonized by atoxigenic strain *Aspergillus flavus* AF36 has been evaluated in small-scale test plots since 1989.<sup>81</sup> Strain seeding has caused large and significant changes in the *A. flavus* population on the crop and in the soil. Applications have resulted in the applied atoxigenic strain becoming dominant in the field and aflatoxin-producing strains becoming less frequent.<sup>83</sup> These changes in the *A. flavus* populations have been associated with very significant reductions (75 to 99%) in aflatoxin contamination.<sup>83</sup> An inverse relationship between the incidence of the applied strain on seed from treated and control plots and the concentrations of aflatoxins in the seed has been well established (Figure 14.3).

*A. flavus* typically becomes associated with crops in the field during crop development. Field plot tests indicate that atoxigenic strain applications do not

increase the amount of *A. flavus* on the crop at maturity or the percent of the cottonseed crop infected by *A. flavus*.<sup>83</sup> Both toxigenic and atoxigenic *A. flavus* that become associated with the crop in the field remain with the crop during harvest, storage, and processing.<sup>91</sup> Thus, crop vulnerability to aflatoxin contamination remains until the crop is ultimately processed or consumed, and atoxigenic strains seeded into agricultural fields prior to crop development continue to remain with the crop, providing long-term postharvest protection.<sup>91</sup> Atoxigenic strains applied both prior to harvest and after harvest have been shown to provide protection from aflatoxin contamination in corn,<sup>80</sup> even when toxigenic strains are associated with the crop prior to application.

## B. SUITABILITY OF BIOLOGICAL CONTROL STRATEGY

Economics of aflatoxin contamination will probably dictate the regions in which atoxigenic strains are utilized. The current projected cost to produce materials by a grower cooperative for atoxigenic strain applications is expected to be \$5/acre or less. If treatments are 70% effective and an average of 40 to 70% of seed is above 20 ppb aflatoxins and the benefit of having aflatoxin-free seed is \$20 to \$40 a ton, then growers will gain an average return above an initial \$5/acre investment of \$0.60–\$14.60/acre in regions where one ton of cottonseed is produced per acre. In severe aflatoxin years, even with a 90% reduction in contamination, growers may achieve no economic gain because the resulting crop will still contain over 20 ppb aflatoxin B<sub>1</sub>. In certain regions, cottonseed crops containing over 1000 ppb are not uncommon.<sup>92</sup> Benefits could also arise from the applied atoxigenic strains remaining with the crop until use and thus preventing increased contamination during transit and in storage at dairies and reducing litigation. Economics might be improved by both long-term and cumulative benefits resulting from the strain's ability to remain in fields until the next crops are planted. Further, field-plot tests have demonstrated that atoxigenic strain applications have a long-term influence on *A. flavus* populations resident in agricultural fields.<sup>93,94</sup> Thus, atoxigenic strain applications have benefits over multiple seasons, and long-term, area-wide changes in the aflatoxin-producing potential of *A. flavus* populations can be achieved.

Just as dust doesn't stay in the field where it was raised, fungi do not stay in the field to which they were applied. Over time, therefore, applications could reduce contamination in an area as a whole. This should facilitate the development of either gin-wide or community-wide management programs. In areas where multiple crops are affected by contamination (e.g., corn, cotton, and peanuts), treatments to one crop could possibly benefit all crops. Nonetheless, a better understanding of the long-term benefits of atoxigenic strain applications are needed prior to development of area-wide management programs based on this technology.

Tests to evaluate the longevity of changes to *A. flavus* communities induced by atoxigenic strain applications to commercial cotton fields in Arizona have been conducted since 1996. The experimental program included treatments over a 3-year period (1120 acres total) and monitoring the *A. flavus* community from 1996 through 1999.<sup>84,93</sup> Both a scaled-up laboratory procedure for producing inoculum<sup>88</sup> and a quality control program were approved by the Environmental Protection Agency

(EPA) for use under Experimental Use Permit 69224-1. During this period, over 11,000 lb of colonized wheat were produced in the laboratory. The product was shipped without refrigeration to growers in food-grade, 5-gallon polyethylene buckets and was stored on-farm without special care until use. Different treatment regimes were applied to different fields, with some fields receiving treatment only in a single year and others receiving treatments in multiple years.<sup>94</sup>

Sterile wheat seed colonized by an atoxigenic strain was applied to 22 fields ranging in size from 10 to 160 acres from 1996 to 1998. The material was applied either by air or on the ground at the rate of 10 lb per acre. In order to monitor changes to the composition of *A. flavus* populations, soil samples were collected prior to application each year. From 1996 through 1999, over 8000 isolates of *A. flavus* were cultivated from soil samples taken from the treatment areas. Isolates were characterized by strain, and those assigned to the L strain of *A. flavus* were further characterized by vegetative compatibility analysis in order to determine applied strain distribution.<sup>94</sup>

One year after application, atoxigenic strain incidence was greatly increased, and incidence of the highly toxigenic S strain was greatly decreased in treated and adjacent fields.<sup>95</sup> The applied strain incidence gradually declined by the second year after application; however, even with this decline, the atoxigenic strain remained in treated fields at levels significantly higher than prior to treatment. The incidence of the applied strain in fields adjacent to treated fields 1 and 2 years after application was variable.<sup>95</sup> Thus, when planning area-wide management, possible directional movement of the applied strains from treated to untreated fields should be considered. Crop and crop stage, not only in treated fields but also in nearby untreated fields, at application are important determinants of the extent to which long-term and area-wide benefits are achieved.

Results of initial field studies have allowed the Arizona Cotton Growers Association to pursue research and development of an area-wide aflatoxin management program utilizing atoxigenic strains of *A. flavus* as a central component.<sup>96</sup> The Arizona Cotton Research and Protection Council has taken the lead in partnership with the Agricultural Research Service to develop an effective management program and to establish a grower-owned facility for the production of commercially useful quantities of atoxigenic strain inoculum. The Arizona growers applied in 1998 to the EPA for an atoxigenic strain registration that would permit treatment of all cotton in Arizona. In 1999, an expansion of the Experimental Use Permit was received that allowed treatment of up to 20,000 acres per annum.<sup>96</sup> Over 10,000 acres were treated in 1999. Improvements to the manufacturing facility were expected before treatment of the year 2000 crop. Once the Arizona growers have a facility that can produce the quantity and quality of material required, they will have to undergo a development series during which the agronomic practices associated with atoxigenic strain use are optimized to achieve both single-season benefit and long-term, area-wide reduction in the aflatoxin-producing potential of fungi resident in Arizona's agricultural areas.

Selection and seeding of fungal strains could, ultimately, become a useful tool in reducing the vulnerability of all crops grown in treated areas to aflatoxin contamination. Such selection and seeding of fungal strains may also be useful for other

fungi that either cause contamination problems or provide needed activities in the agroecosystem. Some management of or modification to the local fungal strains may be required to enhance effectiveness in different geographical regions. Development of methodologies to specifically genetically modify *A. flavus* communities offers the opportunity to exert an unprecedented control over the safety and benefits of the filamentous fungi in the agricultural environment. This extends beyond the use of our extensive knowledge of the regulation of aflatoxin biosynthesis to construct specific gene-disrupted strains without the ability to produce aflatoxins. This potential extends to the development of "designer microbes" ideally suited to the environment in which they must compete and with safety features that may preclude animal pathogenesis or allergenicity.

## VII. ENHANCEMENT OF HOST-PLANT RESISTANCE TO THE AFLATOXIN CONTAMINATION PROCESS

Traditional breeding or genetic engineering of plants with genes expressing either resistance against fungal infection or inhibition of aflatoxin biosynthesis most likely would achieve significant control of aflatoxin contamination. This is especially true for corn, which possesses a large amount of natural genetic diversity with respect to fungal infection. Naturally resistant crop germplasm, if identified, provides us not only with a source of resistance but also nature's lesson as to the specific requirements of resistance (e.g., antifungal compounds, regulation of these compounds, and physiological conditions for bioactivity). Studies in which various crops have been inoculated with aflatoxin-producing fungi have shown clearly (particularly in corn) that resistance mechanisms exist; however, our knowledge about resistance is incomplete, and little is known about the specific genetic and/or biochemical traits required for the expression of significant resistance against the aflatoxin contamination process.

Research efforts focusing on the development of crop germplasm with resistance to insect injury and effects of drought, acting along with resistance against fungal growth and aflatoxin production, could prove beneficial. In corn, the focus has included: (1) indirect protection of developing kernels by husk cover and anti-nutritional substances in silks, (2) direct protection provided by kernel compounds that block fungal development, and (3) direct protection provided by external integuments of developing kernels.<sup>97</sup> Investigations have also centered on identification of resistance to drought stress and insect damage as useful and obtainable traits.<sup>97</sup> Insect damage is often positively correlated with aflatoxin contamination; however, insects probably play a more important role in the infection process of *A. flavus* when conditions are less favorable for the fungus.<sup>98</sup> Under conditions more favorable to *A. flavus* (high temperatures, drought stress), the role of insect injury in aflatoxin contamination probably diminishes.<sup>98</sup> *A. flavus* may even display limited parasitic abilities under such conditions stressful to the plant.<sup>99</sup> In cotton, pink bollworm damage has been closely associated with aflatoxin contamination, and resistance to pink bollworm was thought to be a potential solution to the cottonseed aflatoxin contamination problem.<sup>100</sup> Early field-plot results with transgenic Bt cottons indicated that these

cottons were both highly resistant to pink bollworm and to aflatoxin contamination.<sup>101</sup> When commercial crops were evaluated, the transgenic Bt cultivars continued to express very strong, sustained resistance to the pink bollworm; however, Bt cottonseed lots highly contaminated with aflatoxins were rapidly identified,<sup>101</sup> and in some locations differences in aflatoxin contamination between transgenic Bt and conventional cultivars were not seen in the commercial crop.<sup>91,92,101</sup> This may be true for corn, as well.<sup>102</sup>

Current research efforts are primarily focused on kernel/seed morphologic and chemical (e.g., antifungal protein) resistance to fungal infection. The resistant genotypes generally inhibit aflatoxin production indirectly, through the inhibition of fungal growth.<sup>73,103,104</sup>

## A. CURRENT PROGRESS IN PLANT BREEDING STRATEGIES

### 1. SCREENING TECHNOLOGIES

Screening crops for resistance to kernel or seed infection by *A. flavus* or for resistance to aflatoxin production is a more difficult task than most disease screening. Successful screening in the past<sup>105</sup> has been hindered by the lack of: (1) a resistant control, (2) inoculation methods that yield infection/aflatoxin levels high enough to differentiate among genotypes (natural infection is undependable), (3) repeatability across different locations and years, and (4) rapid and inexpensive methods for assessment of fungal infection and aflatoxin levels. Several plant inoculation methods for assessing crops such as corn and cotton for resistance to *A. flavus* invasion and subsequent aflatoxin contamination have been tried with varying degrees of success — for example, the pinbar inoculation technique for inoculating corn kernels through husks with the fungal conidia, the silk inoculation technique in corn, infesting corn ears with insect larvae infected with *A. flavus* conidia, wound inoculation of cotton bolls or tree nuts to mimic the exit holes of insects, etc.<sup>106-109</sup> Amending soils containing developing peanuts has also been examined for assessing resistance to *A. flavus* infection.<sup>110,111</sup>

#### a. Corn

Screening studies to evaluate various crop genotypes for resistance to aflatoxin contamination have demonstrated differential levels of resistance in the crops, but the genotypes all showed only partial resistance. However, two resistant corn inbreds (Mp420 and Mp313E) were discovered and tested in field trials at different locations and released as sources of "resistant" germplasm.<sup>112,113</sup> The pinbar inoculation technique (a precision kernel-wounding technique) was one of the methods employed in the initial trials, and contributed towards the separation of partially resistant from susceptible lines.<sup>112</sup> Several other corn inbreds, demonstrating partial resistance to aflatoxin contamination in Illinois field trials (employing a modified pinbar technique), also were discovered.<sup>114</sup> Another source of partially resistant germplasm is the corn-breeding population GT-MAS: gk, which was derived from visibly classified segregating kernels obtained from a single fungus-infected hybrid ear.<sup>115</sup> It tested resistant in trials conducted over a 5-year period, where a kernel knife inoculation technique was employed. These discoveries of partially resistant germplasm may

have been facilitated by the use of inoculation techniques capable of repeatedly providing high infection/aflatoxin levels for genotype separation to occur. These corn lines do not generally possess commercially acceptable agronomic traits; however, they may be sources of resistance genes and, as such, provide a basis for the rapid development of host resistance strategies to eliminate aflatoxin contamination.

#### b. Peanuts

Several sources of resistant peanut germplasm have also been identified from a core collection representing the entire peanut germplasm collection.<sup>110</sup> Over 95% of this core has been preliminarily screened in a single environment: 16 genotypes tested over 3 years in two environments still display low levels of aflatoxins. A possible link between low linoleic acid content in peanuts and low preharvest aflatoxin production has been indicated.<sup>110</sup> Significant correlations have also been observed between leaf temperature and aflatoxin levels and/or visual stress ratings and aflatoxin levels. The preliminary screening of peanut genotypes using either or both of these traits could greatly reduce expenses involved in developing resistant cultivars. The promising germplasm, however, has less than acceptable agronomic characteristics and is being hybridized with those with commercially acceptable features. Resistant lines are also being crossed to pool resistances to aflatoxin production. To date, some success has been achieved in identifying resistant peanut germplasm, and field studies are being conducted by various researchers to verify this trait. Methods to improve screening of peanuts for resistance to *A. flavus* have been developed. A system of evaluating peanuts in the field through the manipulation of drought stress was successfully tested.<sup>116</sup> Also, an *in vitro* seed culture system demonstrating water stress responses in peanuts, similar to field responses, and variations in peanut phytoalexins and aflatoxin levels appears potentially useful.<sup>111,112</sup>

#### c. Tree Nuts

Among tree nuts, strategies for controlling preharvest aflatoxin formation by breeding for host resistance have been mostly studied in almonds and walnuts.<sup>118-120</sup> The approach employed in this effort is to integrate multiple genetic mechanisms for control of *Aspergillus* spp. as well as Navel orangeworm (*Paramyelois transitella* Walk), which appears important for initial fungal infection. Resistance to fungal colonization by incorporating seed coat resistance to infection is being pursued; however, genotypes demonstrating inhibition to fungal infection in seed tissues have been inconsistent over different environments.<sup>118</sup> Studies have also been conducted with figs and pistachios to identify the mode of infection of these crops by *A. flavus*. Once this parameter is clearly understood, strategies could be developed to identify germplasm with agronomically desirable characteristics and resistance to fungal infection.<sup>120,121</sup>

Generally speaking, crop varieties showing reduced levels of aflatoxin contamination have been produced by plant breeding; however, unacceptable levels of toxin still resulted when the plants were exposed to severe environmental pressures. Hindrances to resistance screening, discussed above, certainly played key roles in this lack of success.<sup>112</sup> Screening for resistance can produce useful results, but specific resistance traits or markers must be measured before appropriate breeding

and selection techniques can be properly exploited.<sup>122</sup> Plating kernels or seed to determine the frequency of fungal infection or examining corn kernels for emission of a bright greenish-yellow fluorescence (BGYF) are methods that have been used for assessing *A. flavus* infection.<sup>105</sup> While these methods can indicate the presence of *A. flavus* in seed, neither can provide accurate quantitative or tissue-localization data useful for effective resistance breeding. Several other protocols have been developed and used for separation and relatively accurate quantitation of aflatoxins,<sup>123,124</sup> but fungal growth quantitation has not been possible until recently, with the genetically engineered fungi containing a reporter gene as a growth indicator.

## 2. Novel Screening Methods To Better Assess Fungal Infection and Growth

A laboratory kernel screening assay (KSA) has been developed and used to study resistance to aflatoxin production in GT-MAS:sk corn kernels.<sup>125</sup> KSA is an inexpensive humidity chamber technique in which kernels are screened at 100% humidity and 31°C, conditions that favor *A. flavus* growth and aflatoxin production.<sup>73</sup> Data from KSA experiments can be obtained 2 weeks after experiments are initiated. KSA experiments have confirmed GT-MAS:sk resistance to aflatoxin production and have demonstrated that the resistance is maintained even when the pericarp barrier, in otherwise viable kernels, is breached by wounding.<sup>125</sup> The wounding experiment facilitates both differentiation between different resistance mechanisms and comparison with other traits (e.g., fungal growth, protein induction). Apparently, there are two levels of resistance: one at the pericarp and another at the subpericarp level. KSA studies have demonstrated that the pericarp wax composition influences the kernel resistance phenotype<sup>103,104,126</sup> and that there are quantitative and qualitative differences in pericarp wax between GT-MAS:sk and susceptible genotypes.<sup>127</sup>

Kernel screening assays have also confirmed sources of resistance among inbreds tested in Illinois field trials.<sup>73,114</sup> When selected resistant Illinois inbreds (M182, C12, and T115) were examined by the modified KSA, which included an *A. flavus*-GUS transformant (a strain genetically engineered with a gene construct consisting of a  $\beta$ -glucuronidase reporter gene linked to an *A. flavus*  $\beta$ -tubulin gene promoter for monitoring fungal growth), a positive relationship between the degree of fungal infection and aflatoxin levels was established.<sup>73,128,129</sup> Moreover, kernel resistance to fungal infection in nonwounded and wounded kernels was clearly demonstrated visually and quantitatively.<sup>129</sup> Thus, it is now possible to accurately assess fungal infection levels and to determine if a correlation exists between infection and aflatoxin levels in the same kernels. *A. flavus* GUS transformants with the reporter gene linked to an aflatoxin biosynthetic pathway gene could also provide a quick and economical way to indirectly measure aflatoxin levels.<sup>129,130</sup>

The kernel screening assay has several advantages to complement traditional breeding techniques:<sup>114,125</sup> (1) it can be performed and repeated several times throughout the year and outside of the growing season; (2) it requires few kernels; (3) it can detect/identify different kernel resistance mechanisms; (4) it can dispute or confirm field evaluations (e.g., identify escapes); and (5) it can correlate findings and inoculations in the field. Field trials are irreplaceable for confirmation of



*A. flavus*: (2) gene promoters also must be selected that will allow appropriate expression of antifungal genes, at a desired time, in the candidate crop; and (3) genetic transformation needs to be adapted to each specific crop.<sup>142</sup> Resistance to *A. flavus* infection in plants could consist of an interaction of multiple components and biochemical changes that are either preformed or induced upon past invasion. Stimulation by elicitors may result in changes in gene expression and induction of pathogenicity-related (PR) proteins.<sup>143-145</sup> Multiple genes governing constitutive and inducible metabolic factors/mechanisms may exist and be expressed optimally at different stages of seed/kernel maturation. Development of optimal host-plant resistance through genetic engineering will require a sound understanding of the multiple factors that endow a plant with resistance to fungal attack.

#### B. CANDIDATE ANTIFUNGAL COMPOUNDS

Identifying resistance (e.g., in corn) makes it possible to correlate resistance with many endogenous small molecular weight compounds and biomacromolecules in kernel tissues already implicated as antifungal at various stages of kernel development in grain crops.<sup>146-152</sup> However, compounds with activity against other fungal species are ineffective against *A. flavus*, thus it is important to select the best candidate genes for these inhibitory compounds before plant genetic engineering procedures are initiated. A list of candidate antifungal compounds includes RIPs, lectins, relatively small molecular weight polypeptides, cell-surface glycoproteins, hydrolases, and certain basic proteins.<sup>153</sup> For example, the 14-kDa trypsin inhibitor,<sup>139</sup> shown to be correlated with kernel resistance to *A. flavus* infection of corn, when expressed in transgenic tobacco greatly enhances resistance to the tobacco pathogen, *Colletotrichum destructivum* (see details below).<sup>154,155</sup> A putative peptide also has been partially purified from aqueous kernel extracts of resistant inbred, Tex6, which demonstrated antifungal activity against *A. flavus*.<sup>156</sup> The corn kernel pathogenesis-related (PR) proteins appear to have a function during the normal process of seed germination;<sup>144,147,148,150</sup> however, they are induced to accumulate in response to fungal infection, and their expression is tissue-specific.<sup>144,157</sup> A further investigation of the kernel PR proteins using resistant and susceptible genotypes to examine specific tissue expression of these proteins under varying kernel physiology may facilitate the isolation of factors responsible for subpericarp resistance.

A number of potentially useful antifungal enzymes/proteins are produced either constitutively or in response to fungal attack in plants. These include chitinases and  $\beta$ -1,3-glucanases,<sup>152,158-160</sup> osmotins,<sup>161</sup> protease inhibitors,<sup>162</sup> and polygalacturonase inhibitor proteins (PGIPs).<sup>163</sup> In addition, small molecular weight peptides have been isolated from organisms other than plants that also show promise as antifungal agents — for example, the cecropins<sup>164</sup> and magainins<sup>165</sup> of insect and amphibian origins, respectively, and their synthetic analogs.<sup>166,167</sup>

Several recent studies have suggested the potential of manipulating/inducing the lipoxygenase (LOX) pathway in plants to ward off fungal attack.<sup>165</sup> *A. flavus* exhibits strong lipolytic activity during infection on oilseeds<sup>169</sup> and at times causes substantial deterioration of the crop seeds and oils it has contaminated. Lipase activity originating from fungal degradation of host-plant membrane tissues releases fatty acids

resistance; however, the KSA may eliminate many preliminary field screenings and facilitate an in-depth investigation of kernel responses to fungal infection and aflatoxin production.

Using these new technologies, the fungus can now be "tracked" during its invasion process in various kernel/seed compartments. Studies employing KSA,<sup>121</sup> as well as other techniques,<sup>121</sup> have demonstrated that kernel embryos are colonized before endosperm tissue is invaded by aflatoxin-producing fungi. Embryo viability has also been shown to be necessary for the expression of kernel resistance.<sup>122</sup> It is possible that resistance, especially subpericarp, is a function of the kernel's ability to limit fungal colonization to a small area after wounding. Limiting fungal ingress may help prevent fungal spread through the kernel and interruption of whole-kernel expression of embryo-based resistance mechanisms, thus denying easy access of the fungus to a substrate most conducive to aflatoxin production.<sup>128,129</sup> The high levels of aflatoxins detected in susceptible kernels have often been considered the primary result of fungal metabolic activity on an embryonic substrate; however, there is evidence that these high levels of aflatoxin production may result from later fungal activity in the endosperm.<sup>132</sup>

The reporter gene constructs, when placed under the control of specific aflatoxin pathway gene promoter, can be utilized to identify environmental and nutritional signals in the plants and their sites of synthesis in specific plant tissues, which in turn are important in governing the degree of aflatoxin production. Further experiments can now be conducted to: (1) elucidate how environmental factors (fungal growth substrates, host plants, etc.) influence genetic regulation of aflatoxin biosynthesis; (2) utilize reporter gene assays to assess the influence of plant biochemicals on aflatoxin gene expression during the host plant *A. flavus* interaction; and (3) determine the effect of selected plant volatiles derived from the plant lipoxygenase pathway on fungal development, reproduction, and sporulation, processes critical to fungal survival and sharing genetic connection with the aflatoxin biosynthetic process.

### 3. Identification of Resistance Markers and Their Functions in Crops Vulnerable to Aflatoxin

A great preponderance of resistance "markers" potentially of value in plant breeding for resistance to the aflatoxin contamination process have been discovered in corn. In other crops, resistance markers that can be utilized in breeding to select for resistant progeny have proven more difficult to identify. The possible link between low linoleic acid content in peanuts and low preharvest aflatoxin production was suggested, but after further analysis the nature of this relationship has proven to be elusive. Progress has been made in identifying chromosome regions in corn associated with resistance to *A. flavus* infections and inhibition of aflatoxin production in corn using RFLP analysis in three resistant lines (R001, LB31, and Tex6), after mapping populations were developed using B73 and/or Mo17 elite inbreds.<sup>133,134</sup> In some cases, chromosomal regions were associated with resistance to *Aspergillus* ear rot but not with aflatoxin inhibition, and vice versa, whereas other chromosomal regions were found to be associated with both traits. This suggests that these two

traits may be at least partially under separate genetic control. Also, it was observed that variation can exist in the chromosomal regions associated with *Aspergillus* ear rot and aflatoxin inhibition in different mapping populations, suggesting the presence of different resistance genes in different resistant germplasm. RFLP may provide the basis for employing a successful strategy of pyramiding different types of resistances into commercially viable germplasm, while avoiding the introduction of undesirable traits. However, there is a real need to identify specific traits at the gene level associated with the above RFLP markers and assign a specific biochemical or other function to the trait. This information is needed in order to make decisions on which traits to include to pyramid resistance through traditional plant breeding or transfer specific resistance traits into crops vulnerable to aflatoxin contamination by genetic engineering.

Studies demonstrating subpericarp (wounded-kernel) resistance in corn kernels have led to research with the aim of identifying subpericarp resistance mechanisms. When kernels of susceptible genotypes were allowed to imbibe water at 100% humidity at 31°C for 3 days prior to being subjected to the KSA protocol, aflatoxin levels were drastically and significantly reduced compared to unimbibed controls.<sup>103,104</sup> Kernel proteins induced during imbibition may have inhibited growth and/or fungal elaboration of aflatoxins. Examinations of kernel proteins of several genotypes revealed several differences between resistant and susceptible genotypes.<sup>135</sup> In imbibed susceptible kernels, decreased aflatoxin levels were associated with germination-induced synthesis of ribosome inactivating protein (RIP) and zeamain.<sup>136</sup> Both zeamain and RIP have been demonstrated to inhibit *A. flavus* growth *in vitro*.<sup>136</sup> These studies implicate proteins as potentially involved in kernel resistance to *A. flavus* infection and aflatoxin production.

Two kernel proteins have been identified from resistant inbred Tex6 which may contribute to resistance to aflatoxin production.<sup>137</sup> The 28-kDa protein inhibits *A. flavus* growth, while a 100-kDa protein inhibits toxin formation with little effect on fungal growth. With the recent elucidation of aflatoxin biosynthetic pathway genes and enzymes and regulatory mechanisms (see earlier section on aflatoxin biosynthesis),<sup>118</sup> technology could be developed with the potential for identifying seed/kernel resistance mechanisms that directly inhibit aflatoxin biosynthetic activity. An examination of kernel protein profiles of 13 corn genotypes revealed that a 14-kDa trypsin inhibitor protein is present at relatively high concentrations in seven resistant genotypes, but is present only in low concentrations in six susceptible ones.<sup>119</sup> This protein exhibits strong bioactivity against the growth of *A. flavus*, *A. parasiticus*, and a morphologically diverse group of other fungi.<sup>128,129,140,141</sup> Thus, comparisons of kernel protein profiles between susceptible and resistant genotypes may shorten the time it takes to identify resistance-associated proteins to be used in marker-assisted breeding.

#### 4. Genetic Engineering Strategies

Several prerequisites must be met in order to employ genetic engineering as a means of developing host resistance against aflatoxin-contamination in crops: (1) resistance genes, native or foreign, should be identified that express inhibitory activity against

from bound triglycerides and triggers the LOX-hydroperoxide lyase (HPLS) enzyme pathway, converting linoleic and linolenic acids into hexenal and *cis*-3-hexenal, respectively.<sup>170</sup> Then, *cis*-3-hexenal is usually isomerized to *trans*-2-hexenal, both enzymatically and nonenzymatically.<sup>170</sup> Recently, it has been reported that specific LOX decay products, such as 13S-hydroperoxy fatty acid, jasmonic acid, and C<sub>8</sub>-C<sub>10</sub> alkenals and alkanals, may function as important signal molecules in host-pathogen interactions.<sup>32,76,117,171,172</sup> Jasmonic acid has also been shown to inhibit aflatoxin production and delay spore germination of *A. flavus*.<sup>173</sup> The antifungal properties of small chain alkanals and alkenals (derived from the LOX pathway) produced by cotton leaves have been demonstrated in solid and liquid cultures of aflatoxigenic *Aspergillus* spp.<sup>30,75,174</sup> Because of the mode of activation of these volatile aldehydes and because of the significant antifungal activity they exhibit, these compounds could function as "gaseous phytoalexins" in the cotton plant.<sup>75</sup>

There are only a few candidate genes whose expression products demonstrate convincing inhibitory activity against *A. flavus* and show promise for transformation of plants to reduce infection of seed by this particular fungal species. Included among these antifungal products are certain small lytic peptides. It is relatively easy to chemically synthesize genes encoding small peptides using an oligonucleotide synthesizer for transformation of plants, as only relatively small coding regions are required for their complete synthesis. Cecropins, for example, are lytic peptides of 22 to 23 amino acids in linear arrays that comprise antimicrobial systems found in insects and pig intestine.<sup>164,175</sup> The broad antibacterial activities of cecropins are due to the formation of large pores in the cell membrane.<sup>176,177</sup> They apparently do not lyse erythrocytes or other higher eucaryotic cells<sup>178</sup> but do inhibit growth of *A. flavus* mycelia.<sup>179</sup> Rajasekaran et al.<sup>155</sup> reported that a synthetic lytic peptide when transformed into tobacco greatly enhances resistance to *Colletotrichum destructivum*. In addition to lytic peptide genes, a variety of other candidate antifungal genes from bacterial, plant, and mammalian sources, have a good potential to be active against *A. flavus* upon transformation into plants. Genes encoding LOX are available from plant sources.<sup>180,181</sup> The LOX products such as 13-hydroperoxylinoleic acid and its breakdown products/volatiles, such as hexenal and hexanal, are antifungal and interfere with the aflatoxin pathway. Genes encoding for haloperoxidases are also available for possible genetic engineering of plants for antifungal resistance.<sup>182,183</sup> In bioassays using *A. flavus* as the test organism, addition of a myeloperoxidase greatly enhanced (90-fold) the lethality of H<sub>2</sub>O<sub>2</sub> by catalyzing its conversion to sodium hypochlorite.<sup>184</sup> A bacterial chloroperoxidase also greatly reduced the viability of *A. flavus* conidiospores.<sup>185</sup> H<sub>2</sub>O<sub>2</sub> is induced in plants by wounding or injuring of plant tissues, a process often associated with pest attack, thus the substrate for these unique peroxidases should be available in the specific host-plant tissues under attack.<sup>185</sup>

### C. GENE PROMOTERS

Promoter elements that allow constitutive, wound-inducible, or tissue-specific expression of antifungal genes in plants have been identified. Characterized promoter elements that are useful in obtaining optimum expression of antifungal genes in

plants include the CaMV 35S<sup>186</sup> and ubiquitin 3 promoter elements (constitutive),<sup>187</sup> the protease inhibitor II promoter (wound-inducible),<sup>188</sup> and storage protein gene promoters (seed-specific expression).<sup>189</sup> Peanuts have successfully been transformed with a wound-inducible promoter from soybean vegetative storage protein (*vsp*).<sup>190</sup> When the *vsp*-promoter/GUS gene fusion is inserted into peanut, expression of the GUS gene follows temporal and spatial patterns as would be predicted from soybean.<sup>190</sup> The *E. coli*  $\beta$ -glucuronidase reporter gene has been used to assess the level of gene expression obtained under the control of some of the above promoters in transformed cotton.<sup>142,191</sup>

#### D. TRANSFORMATION METHODS

Antifungal genes in suitable gene expression vectors have been used in the transformation of plants by a variety of methods. The two most common methods include *Agrobacterium*-mediated gene transfer<sup>192,193</sup> and biolistic particle delivery or "gene gun" technology.<sup>142,155,167,191,194</sup> After gene transfer, transformed tissues are identified by growth on selective medium, and whole plants are regenerated from the selected, transformed cells. Cotton transformation has been accomplished using the *Agrobacterium*-based system and cotton hypocotyl sections, although the subsequent regeneration procedure in this system is not necessarily straightforward and can be lengthy. Cotton regeneration from transformed hypocotyl tissue involves the development of transformed embryogenic cell lines, embryoid formation, dissection, desiccation of embryos, and subsequent germination of the embryos.<sup>195,196</sup> Coker cultivars 201 and 312 of cotton have been transformed with *Agrobacterium*-mediated systems and regenerated.<sup>197,198</sup> Transformation of other commercially important cotton varieties has proven difficult due to the inability to generate embryogenic cell lines; however, *Agrobacterium*-mediated transformation has been successfully employed on elite Acala and Coker cultivars.<sup>199</sup> Problems, nevertheless, remain regarding the efficiency of this method of transformation and its adaptability to a wide range of germplasm. To circumvent the problem of cultivar-dependent regeneration, investigators have used the biolistic approach to transform cotton.<sup>142,191,200,201</sup> Peanut is also currently being transformed with antifungal genes by microprojectile bombardment of embryogenic tissues.<sup>202</sup> Walnut has been successfully transformed with barley lectin and nettle lectin antifungal genes.<sup>203</sup> The somatic embryo is the targeted tissue in walnut, and a new technique for cryopreservation of walnut somatic embryos is being used for long-term storage of embryo lines to prevent somaclonal variation and loss of lines to contamination.<sup>203</sup>

### VIII. CONCLUSION

Several approaches are being explored and developed using new methods in biotechnology to eliminate pre-harvest aflatoxin contamination of food and feed. These approaches have resulted from recently acquired information about: (1) the ecology and epidemiology of aflatoxin-producing fungi, (2) molecular mechanisms governing aflatoxin biosynthesis, and (3) plant-derived metabolites that inhibit aflatoxin biosynthesis. Experience in our laboratory suggests a combined approach utilizing

both host defense augmentation and biological control will be necessary to complement existing conventional methods in the eventual elimination of aflatoxin from the food and feed supply.

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# Formation of Sclerotia and Aflatoxins in Developing Cotton Bolls Infected by the S Strain of *Aspergillus flavus* and Potential for Biocontrol with an Atoxigenic Strain

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## ABSTRACT

Garber, R. K., and Cotty, P. J. 1997. Formation of sclerotia and aflatoxins in developing cotton bolls infected by the S strain of *Aspergillus flavus* and potential for biocontrol with an atoxigenic strain. *Phytopathology* 87:940-945.

*Aspergillus flavus* can be divided into the S and L strains on the basis of sclerotial morphology. On average, S strain isolates produce greater quantities of aflatoxins than do L strain isolates. Sclerotia of the S strain were observed in commercial seed cotton from western Arizona. Greenhouse tests were performed to better define sclerotial formation in developing bolls. Eight S strain isolates were inoculated into developing bolls via simulated pink bollworm exit holes. All eight isolates formed sclerotia on locule surfaces, and seven of eight isolates produced sclerotia within developing seed. Boll age at inoculation influences formation of sclerotia. More sclerotia formed within bolls that were less than 31 days

old at inoculation than in bolls older than 30 days at inoculation. Frequent formation of sclerotia during boll infection may both favor S strain success within cotton fields and increase toxicity of *A. flavus*-infected cottonseed. Atoxigenic *A. flavus* L strain isolate AF36 reduced formation of both sclerotia and aflatoxin when coinoculated with S strain isolates. AF36 formed no sclerotia in developing bolls and was more effective at preventing S strain isolates than L strain isolates from contaminating developing cottonseed with aflatoxins. The use of atoxigenic L strain isolates to prevent contamination through competitive exclusion may be particularly effective where S strain isolates are common. In addition to aflatoxin reduction, competitive exclusion of S strain isolates by L strain isolates may result in reduced overwintering by S strain isolates and lower toxicity resulting from sclerotial metabolites.

**Additional keywords:** biocompetition, mycotoxins.

Aflatoxins are a group of toxic, carcinogenic fungal metabolites produced by certain isolates of *Aspergillus flavus* Link:Fr., *A. parasiticus* Speare, and *A. nomius* Kurtzman et al. (23). Regulatory limitations on the quantity of aflatoxins permitted in foods and feeds exist throughout most of the world (26). The most toxic and highly regulated aflatoxin is B<sub>1</sub>, which is produced by all three aflatoxin-producing species (20,26). Aflatoxin contamination has long been a concern for the United States cottonseed industry (20), because aflatoxins in contaminated seed can be readily transferred to milk of dairy cows in slightly modified form (19, 22). Cottonseed is a preferred feed for dairy cows, and United States regulations prohibit aflatoxin concentrations over 0.5 µg/kg in milk (15). *A. flavus* is the primary causal agent of aflatoxin contamination of cottonseed. In the United States, aflatoxin contamination of cottonseed is most severe in western Arizona, where contamination is frequently associated with *A. flavus* infection of developing bolls through pink bollworm exit holes (12). Greenhouse techniques to study *A. flavus* infection through simulated pink bollworm exit holes have been developed (5).

On the basis of physiological, morphological, and genetic criteria, *A. flavus* can be divided into two strains, S and L (2,4). Isolates of the S strain produce numerous small sclerotia (<400 µm in diameter) and fewer conidia than L strain isolates. Strain S isolates produce, on average, more aflatoxin than L strain isolates both in culture and within developing cottonseed (4). Many L strain isolates produce little or no aflatoxins (4,13). In Arizona, where afla-

toxin contamination of cottonseed is severe, the S strain is often dominant (9,18). Although several characteristics of the S strain suggest soil adaptation (11), little data on the divergent ecologies of the S and L strains are available. Multiple *A. flavus* strains are known to frequently infect individual locules and seed (1), but interactions between S strain and L strain isolates during seed infection have not been described.

High concentrations of aflatoxins may occur in both conidia and sclerotia of *A. flavus* (28), and certain toxicities associated with *A. flavus* contamination have been attributed to combined activities of aflatoxins and other metabolites present in sclerotia (14,28,29). Over the past 7 years, we have occasionally observed sclerotia of the S strain of *A. flavus* on surfaces of "tight locks" (cotton locules that do not open fully) in commercial cotton in western Arizona. The S strain of *A. flavus* is widely distributed among cotton-producing regions (9). Yet, formation of sclerotia by *A. flavus* within developing crops has been infrequently described (16,24,30), and formation on cotton boll locule surfaces has not been described.

Certain L strain isolates effectively reduce aflatoxin B<sub>1</sub> levels in cottonseed when coinoculated with aflatoxin-producing isolates (6). These atoxigenic strains reduce contamination by competitively excluding aflatoxin-producing isolates during crop infection (10). Atoxigenic strains are being developed as biological control agents directed at preventing aflatoxin contamination (7,8). However, the efficacy of atoxigenic L strain isolates in limiting aflatoxin contamination of cottonseed caused by S strain isolates has not been detailed. Furthermore, it is unknown if atoxigenic L strain isolates can interfere with sclerotial formation by S strain isolates and, in so doing, further reduce seed toxicity by also lowering the incidence of toxins of sclerotial origin.

Boll age at inoculation influences aflatoxin formation in developing cottonseed (5) and may similarly affect the ability of *A. flavus* S strain isolates to colonize cotton locules and produce scler-

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rotia within colonized plant tissue. Such influences may dictate the relative importance of S strain sclerotia to the epidemiology of aflatoxin contamination in the field.

The current study describes, for the first time, sclerotial formation by S strain isolates on locule surfaces and within developing cottonseed. Efficacy of an atoxigenic L strain isolate in limiting both aflatoxin and sclerotial formation by S strain isolates during coinfection of cotton bolls is also described.

## MATERIALS AND METHODS

**Media and cultures.** *A. flavus* S strain isolates from agricultural soils collected in Arizona (AF12, AF65, D2-9, MR3-15, MR5-23, PM3, YV1-1, and YV5-12), Alabama (AL3-39), Louisiana (LA2-5), and Mississippi (STV4-28) were used. L strain isolates (D2-18, PM11, WHT-3, and YV1) used in the aflatoxin production study were isolated from agricultural soils collected in Arizona. Isolate AF36 was isolated from cottonseed collected from the Yuma Valley of Arizona (4). Cultures were maintained in the dark at 31°C on a medium containing 5% V-8 juice and 2% agar (4). For long-term storage, plugs (3 mm in diameter) of sporulating cultures were placed in 12-ml vials containing 5 ml of distilled water and refrigerated at 8°C (5). Inoculum was prepared by suspending conidia from 7- to 10-day-old cultures in distilled deionized water.

**Infection of cotton bolls and developing cottonseed.** Plants of *Gossypium hirsutum* L. (Deltapine 90) were grown in a greenhouse in 3-liter pots containing a 1:1:1 mixture of Pro-mix (Premier Brands, Inc., New Rochelle, NY), coarse sand, and clay loam topsoil. Temperature ranged from 28 to 36°C, and supplemental lighting (400-W General Electric Lucalux bulbs; GTE Corp., Stamford, CT) was applied for 3 h (6 p.m. to 9 p.m.) daily. After 21 days, each plant was fertilized weekly with approximately 500 ml of nutrient solution containing 4 ml/liter of Peter's fertilizer (20-20-20, N-P-K; W. R. Grace & Co., Allentown, PA). One application of Peter's Soluble Trace Element Mix (W. R. Grace & Co.) was administered immediately after planting. Plants were sacrificed 45 days after planting.

Developing cotton bolls were inoculated through simulated exit holes of the pink bollworm as previously described (5). Flowers were tagged and dated at opening. In all experiments, bolls were wounded (1 to 2 mm deep) in a single locule with a cork borer (3 mm in diameter) and inoculated by placing a 10- $\mu$ l aliquot of conidial suspension (about 2,000 conidia) in the wound. For coinoculation experiments, conidial suspensions (10  $\mu$ l, ~2,000 conidia) of each isolate were applied to the same wound.

To assess influences of *A. flavus* AF36 on sclerotial formation by S strain isolates, bolls 25 to 30 days old were inoculated with either an S strain isolate alone or both an S strain isolate and AF36, simultaneously. In each test, four S strain isolates were tested. Treatments were replicated four times and arranged in randomized complete blocks. Replicates consisted of individual plants bearing one or two bolls.

Two separate greenhouse experiments were conducted to determine if the in vivo influence of *A. flavus* AF36 on aflatoxin production by S strain isolates was similar to the influence of AF36 on aflatoxin production by L strain isolates (6,10). In each experiment, the influence of AF36 on aflatoxin contamination of developing cotton bolls by two S strain and two L strain isolates was evaluated. Comparisons were made between bolls (27 to 32 days old) inoculated with only an aflatoxin-producing isolate and bolls inoculated with AF36 immediately after the aflatoxin-producing isolate. Treatments were replicated four to five times, and plants were arranged on greenhouse benches in randomized complete blocks (one plant per replicate per treatment).

To assess the influence of boll age at inoculation on sclerotial formation by S strain isolates on cotton locule surfaces and in developing cottonseed, cotton bolls at various stages of development (16 to 34 days after flowering) were inoculated. In each of two tests, all bolls (two to five per plant) on four plants were inocu-

lated with one of four S strain isolates. Values from all plants inoculated with the same isolate were averaged to form a replicate value. Each of the two tests had four replicates (four isolates), and different isolates were used in each test. In a third experiment, bolls of varying age (16 to 34 days old) on 20 plants were inoculated with a single S strain isolate (LA2-5).

**Measurement of sclerotia and aflatoxin B<sub>1</sub> in boll tissues.** Bolls were harvested in all experiments 17 days after inoculation and dried in a forced-air oven at 60°C for 72 h. After drying, wound-inoculated locules were separated from adjacent uninoculated locules and stored at room temperature (21 to 28°C) in sealed 20-ml plastic vials until analyzed. After weighing, wound-inoculated locules were evaluated for the presence of sclerotia on locule surfaces using the following rating system: 0 = no sclerotia; 1 = 1 to 25 sclerotia; 2 = 26 to 50 sclerotia; 3 = 51 to 100 sclerotia; 4 = 101 to 250 sclerotia; 5 = 251 to 500 sclerotia; 6 = 501 to 1,000 sclerotia; and 7 = >1,000 sclerotia present. Seeds were then separated from lint by hand and halved longitudinally with a razor blade. The percentage of seed per locule with sclerotia, conidiophores, or both was determined with a dissecting microscope (30X magnification). The number of sclerotia per seed was also recorded.

Aflatoxin B<sub>1</sub> concentrations in inoculated locules were determined as previously described (6). Briefly, intact locules were pulverized and extracted with an 85% aqueous acetone solution. Extracts were purified, partitioned against methylene chloride, and concentrated. Concentrates and aflatoxin standards were separated on thin-layer chromatography plates (silica gel 60, 250  $\mu$ m) by development with diethyl ether-methanol-water (96:3:1). Extracts were either concentrated or diluted to permit accurate densitometry, and aflatoxin B<sub>1</sub> was quantified with a scanning densitometer (model es-390; Shimadzu Scientific Instruments, Inc., Tokyo) (21).

**Statistical analyses.** Analyses were performed with the Statistical Analysis System (SAS Institute, Inc., Cary, NC) and Statistica (StatSoft, Inc., Tulsa, OK). For comparisons among isolates and between bolls inoculated by an S strain isolate alone and those coinoculated with both an S strain isolate and an L strain isolate, plants (one to two bolls each with a single inoculated locule per boll) served as replicates. Prior to statistical analyses, all percent values were transformed to the arcsin of the square root of the percent as recommended by Sokal and Rohlf (25). Values for number of sclerotia per seed and aflatoxin B<sub>1</sub> concentration were square root-transformed and log-transformed, respectively, in order to homogenize the variance among treatments. Analysis of variance was performed on all multiple comparisons prior to mean separation tests. When significant differences were detected, mean values were separated using Fisher's least significant difference test. Pearson product-moment correlations were calculated for the influence of boll age at inoculation on sclerotial formation within seed and on locule surfaces. Values for individual inoculated locules were used for these calculations. For comparisons between bolls greater than 30 days old and bolls less than 31 days old at inoculation, data from tests 1 and 2 were subjected to analysis of variance, in which all locules inoculated with a particular isolate constituted a replicate. For test 3, individual locules inoculated with LA2-5 served as replicates in the analysis of variance.

## RESULTS

**Formation of sclerotia in developing locules.** All S strain isolates produced sclerotia on locule surfaces when inoculated into 25- to 30-day-old bolls via simulated pink bollworm exit holes (Fig. 1, Table 1). Sclerotia formed in clusters on inoculated locule surfaces, closely resembling those observed on tight locules collected from the commercial crop in western Arizona (Fig. 1). The morphologies and sizes of sclerotia were typical for the S strain. Most isolates produced relatively high numbers of sclerotia on locule surfaces, averaging over 100 sclerotia per locule. One isolate, STV4-28,

produced significantly ( $P = 0.05$ ) fewer sclerotia, averaging less than 26 per locule. AF36 produced no sclerotia on either locule surfaces or in developing seed in either test. However, cottonseed formed within locules inoculated with AF36 frequently contained conidia between the seed coat and cotyledon (Table 1).

Seven of eight S strain isolates produced sclerotia within developing seed (Figs. 2 and 3, Table 2). Sclerotia initially formed between the seed coat and the developing cotyledons (Fig. 2) and, in certain seed, progressed to replace most of the cotyledons (Fig. 3).

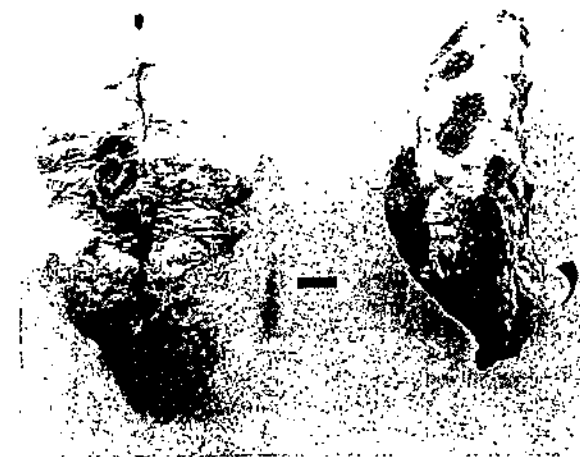


Fig. 1. Locules inoculated with *Aspergillus flavus* S strain isolate LA2-5 alone (right) and in combination with *A. flavus* isolate AF36 (left). Sclerotia only formed on locules inoculated with an S strain isolate. Far fewer sclerotia formed on locules inoculated with both AF36 and an S strain isolate. Bar represents 3 mm.

TABLE 1. Influence of coinoculation with *Aspergillus flavus* AF36 on dry weights of cotton locules inoculated with S strain isolates and on formation of both conidia within developing seed and sclerotia on locule surfaces

Isolate	Locule dry weight (mg)	Sclerotia <sup>a</sup> rating	Seed with conidia (%)
<b>Test 1</b>			
AF36	671.30 b	0.0 c <sup>d</sup>	57 a <sup>2</sup>
AL3-39 (strain S)	602.13 b	4.6 a	0 b
AL3-39 + AF36	739.25 b	1.6 b	56 a
MR3-15 (strain S)	719.25 b	5.9 a	0 b
MR3-15 + AF36	790.25 b	0.4 bc	66 a
LA 2-5 (strain S)	632.50 b	5.5 a	0 b
LA 2-5 + AF36	589.25 b	1.5 b	64 a
YVS-12 (strain S)	783.25 b	5.8 a	0 b
YVS-12 + AF36	699.25 b	0.0 c	75 a
Not inoculated	1,179.13 a	0.0 c	ND
<b>Test 2</b>			
AF36	709.10 bc	0.0 d	54 a
MR5-23 (strain S)	733.13 bc	3.8 a	0 b
MR5-23 + AF36	775.33 bc	2.0 b	54 a
PM 3 (strain S)	593.56 c	3.4 a	0 b
PM 3 + AF36	575.33 c	1.5 bc	46 a
STV4-28 (strain S)	747.75 bc	1.5 bc	0 b
STV4-28 + AF36	645.08 bc	0.4 cd	30 a
YV1-1 (strain S)	580.58 c	4.3 a	0 b
YV1-1 + AF36	810.58 b	0.5 cd	51 a
Not inoculated	1,077.38 a	0.0 d	ND

<sup>a</sup> The number of sclerotia present on the surface of inoculated locules was rated as follows: 0 = no sclerotia; 1 = 1 to 25 sclerotia; 2 = 26 to 50 sclerotia; 3 = 51 to 100 sclerotia; 4 = 101 to 250 sclerotia; 5 = 251 to 500 sclerotia; 6 = 501 to 1,000 sclerotia; and 7 = >1,000 sclerotia.

<sup>b</sup> Values are means of four replicates (locules). Means were compared using Fisher's protected least significant difference test, and values within the same column followed by a common letter are not significantly different ( $P = 0.05$ ). ND = not detected.

In both tests and for all isolates, sclerotia were not produced in most seed from inoculated locules (Table 2). However, in certain seed, large numbers of sclerotia formed, resulting in averages of over 25 sclerotia per seed for five of the eight S strain isolates (Fig. 3, Table 2). Isolates differed in ability to produce sclerotia within developing seed (Table 2). Isolate LA2-5 formed sclerotia within the highest percentage of seed; isolate STV4-28 formed no sclerotia within seed.

In each of the three tests in which bolls of varying age were inoculated with S strain isolates, the number of sclerotia formed on locule surfaces decreased with increasing boll age (Pearson product-moment correlations:  $r = -0.45$  to  $-0.73$ ,  $P < 0.001$ ) (Fig. 4). Although the percentage of seed containing S strain sclerotia also, apparently decreased with boll age at inoculation, this trend was significant for only test 3 (Pearson product-moment correlations:  $r = -0.25$ ,  $-0.39$ , and  $-0.44$ ;  $P = 0.20$ ,  $0.07$ ,  $<0.001$ ). These trends were primarily a result of reduced production of sclerotia in bolls greater than 30 days old at inoculation (Table 3).

Effect of coinoculation with AF36. Coinoculation with AF36 significantly reduced the number of sclerotia formed on surfaces of locules inoculated with S strain isolates (Table 1). Similarly, coinoculation with AF36 frequently reduced both the percentage of



Fig. 2. Formation of sclerotia between a cotyledon and the seed coat. This is the location where sclerotia were most frequently observed. Bar represents 100 µm.



Fig. 3. Cross section of a cottonseed from a locule inoculated with *Aspergillus flavus* S strain isolate LA2-5. Most of the endosperm is replaced by sclerotia. Bar represents 100 µm.

seed containing sclerotia and the number of sclerotia per seed (Table 2). Seed produced in locules coinoculated with AF36 consistently contained *Aspergillus* conidiophores between the cotyledons and seed coat; whereas seed produced both within uninoculated control locules and locules inoculated with an S strain isolate alone contained no conidiophores (Table 1).

Isolate AF36 reduced ( $P < 0.01$ ) aflatoxin B<sub>1</sub> production by the four tested S strain isolates over 99.9% when coinoculated into developing cotton bolls (Table 4). However, AF36 only reduced aflatoxin production by two of the four L strain isolates tested. For those two isolates, reductions of 88 to 96% were achieved.

In both greenhouse tests, inoculation of developing cotton locules resulted in reductions in locule dry weight compared with noninoculated controls (Table 1). The magnitude of dry weight loss in bolls inoculated with AF36 did not exceed losses in bolls inoculated by any of the S strain isolates. Coinoculation of bolls

with AF36 and any of the S strain isolates did not result in further locule weight loss as compared with inoculation with the S strain isolate alone. However, coinoculation of AF36 with isolate YV1-1 resulted in significantly smaller biomass reductions than when YV1-1 was inoculated alone (Table 1).

## DISCUSSION

All of the S strain isolates tested colonized cotton bolls and formed sclerotia on cotton locule surfaces. Seven of eight S strain isolates were also able to form sclerotia within developing seed. In cotton boll inoculation experiments, formation of sclerotia by L strain isolates on locules and in seed has not been observed in either the current or previous (4,5) studies. Similarly, S strain isolates produced sclerotia on pistachio litter, but L strain isolates did not (13). Although sclerotia of *A. flavus* (L strain) have been reported on wound-inoculated corn (30), formation of sclerotia on crops by *A. flavus* is not frequently observed (24). This may stem from both failure to recognize S strain sclerotia and dominance of L strain isolates during boll infection. Waked and Nouman (27) found most aflatoxin within seed that also contained sclerotia when cottonseed collected in Arizona were deliberately stored in the laboratory under conditions favorable to *A. flavus* seed decay. Based on the high toxigenicity of the S strain, its frequent occurrence in Arizona (4,9,18), and the results on sclerotial formation from the

TABLE 2. Influence of coinoculation with *Aspergillus flavus* AF36 on sclerotial formation by *A. flavus* S strain isolates within developing cotton seed

Isolate	Seed evaluated (#)	Seed with sclerotia (%)		Sclerotia per seed (#)	
		Inoculated	Coinoculated	Inoculated	Coinoculated
Test 1					
AL3-39	39, 36 <sup>y</sup>	29 ab <sup>e</sup>	3 c	34.8 ab	1.0 c
MR3-15	46, 38	13 bc	0 c	6.3 bc	0.0 c
LA2-5	34, 49	45 a	2 c	78.6 a	0.1 c
YV5-12	29, 34	26 b	0 c	33.7 ab	0.0 c
Test 2					
MRS-23	56, 67	5 ab	2 b	25.0 ab	20.8 ab
PM3	55, 53	7 ab	2 b	32.3 a	1.0 b
STV4-28	56, 62	0 b	0 b	0.0 b	0.0 b
YV1-1	54, 56	26 a	0 b	6.3 ab	0.0 b

<sup>a</sup> The first value is the number of seed evaluated that were inoculated with the S strain isolate alone (inoculated) and the second value is the number of seeds evaluated that were coinoculated with strain AF36 (coinoculated).

<sup>b</sup> For each parameter, values within a test followed by a common letter are not significantly different ( $P = 0.05$ ) by Fisher's protected least significant difference test. Letters apply to both comparisons within columns and comparisons between columns.

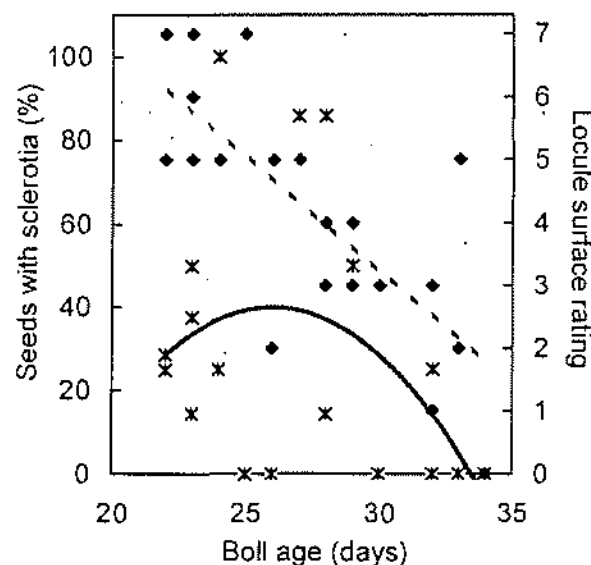


Fig. 4. Relationship between boll age at inoculation and formation of sclerotia by S strain isolate LA2-5 (test 3, Table 4) on locule surfaces (diamonds with dotted) and within developing seed (crosses with solid line). Data points represent values for single locules.

TABLE 3. Sclerotial formation on locule surfaces and within seed of cotton bolls inoculated with S strain isolates of *Aspergillus flavus* either prior to or after 31 days of age

Test <sup>1</sup>	Sclerotial rating on locule surface <sup>a</sup>			Percentage of seed containing sclerotia <sup>1</sup>		
	<31 days old	>30 days old	P <sup>a</sup>	<31 days old	>10 days old	P
1	5.77	2.82	<0.01	38.4	11.4	0.13
2	3.34	0.92	<0.01	10	0	<0.01
3	4.75	2.29	<0.01	35.4	3.6	0.02

<sup>a</sup> The following rating system was used: 0 = no sclerotia; 1 = 1 to 25 sclerotia; 2 = 26 to 50 sclerotia; 3 = 51 to 100 sclerotia; 4 = 101 to 250 sclerotia; 5 = 251 to 500 sclerotia; 6 = 501 to 1,000 sclerotia; 7 = >1,000 sclerotia present.

<sup>b</sup> Percentage of seed within inoculated locules in which sclerotia were observed during dissection and observation under a dissecting microscope (30X).

<sup>c</sup> Values for tests 1 and 2 are means of four replicates; each replicate is the mean for all bolls inoculated with one of four S strain isolates. Different S strain isolates were used in tests 1 and 2. Values for test 3 are means of either seven (>30 days old) or 16 (<30 days old) replicates in which each replicate is the value from a single locule inoculated with LA2-5. In all tests, one locule per boll was inoculated.

<sup>d</sup> Statistical significance of differences between bolls <31 days old and bolls >30 days old at inoculation.

TABLE 4. Influence of *Aspergillus flavus* isolate AF36 on aflatoxin contamination of developing cottonseed by aflatoxin producing isolates of *A. flavus* L strain and S strain

Isolate	Strain	Aflatoxin B <sub>1</sub> (µg/kg)		Reduction (%)
		Alone	Coinoculated	
Test 1				
D2-18	L	52,831 <sup>a</sup>	6,213	88.34 <sup>c</sup>
PM11	L	101,632	3,832	96.23
12	S	177,186	100	99.94
65	S	386,788	19	99.99
Test 2				
WHT-3	L	1,488	4,024	NS
YV1	L	531	0	NS
D2-X	S	91,046	14	99.99
PM3	S	108,640	45	99.96

<sup>a</sup> Values listed are means of five replicates.

<sup>b</sup> Percent reductions are significant ( $P = 0.01$ ) by Fisher's protected least significant difference test except where indicated NS (not significant).

current study, it seems likely that Waked and Nouman (27) were observing contamination caused by the S strain. Sclerotia may be important for overwintering (11) and, as such, formation of sclerotia in developing seed and on locule surfaces may provide an important survival benefit. The S strain, which produces fewer conidia than the L strain (4), may also rely on sclerotia for dispersal. Formation of sclerotia within seed may permit dispersal by herbivorous rodents and birds; formation of sclerotia on locule surfaces is ideal for scattering by modern spindle pickers. Indeed, through dispersal of sclerotia during spindle picking, cotton production may facilitate success of S strain isolates.

Production of sclerotia in developing seed and on locule surfaces varied with boll age at inoculation. Previous studies showed boll age also influences aflatoxin contamination (5). Optimal boll ages at inoculation for both aflatoxin and sclerotial formation are similar, 21 to 32 days after anthesis. Regulation of sclerotial morphogenesis and aflatoxin biosynthesis are interrelated (3) and, thus, it is not surprising that the two coincide. Simultaneous formation of both aflatoxins and sclerotia within cottonseed by *A. flavus* may have both ecological and toxicological significance. Impregnating tissues surrounding sclerotia with aflatoxins may serve to protect sclerotia from insect predation and to prevent utilization of nutritional resources in the seed by competitors (11). Production of sclerotia in the seed may also increase seed toxicity. In addition to aflatoxins, several other highly toxic compounds are known to be concentrated within sclerotia of *A. flavus* (28,29), and synergism in toxicity is known between at least one of these compounds and aflatoxin (14). Furthermore, accumulation of tremorgenic indoloterpenes and other toxic compounds specifically within S strain sclerotia has been described (17). It, therefore, seems likely that S strain sclerotia within commodities cause levels of toxicity beyond that caused by the aflatoxin content alone. Infections by S strain isolates may, thus, cause an unrealized decrease in commodity safety, a decrease not associated with many L strain isolates.

Atoxicogenic L strain isolate AF36 performed as previously described (6,10), by significantly reducing (88 to 99%) the aflatoxin B<sub>1</sub> content of bolls inoculated with six of the eight isolates evaluated. Isolate AF36 also significantly reduced the number of sclerotia formed on locule surfaces and, in some cases, the percentage of seed containing sclerotia. Failure of AF36 to significantly reduce either aflatoxin contamination or sclerotial formation in some treatments probably reflects the great variability in aflatoxin and sclerotial production and not lack of an influence by AF36. AF36 apparently reduced both contamination and sclerotial formation through competitive exclusion as previously described (10). This conclusion is supported by formation of equal quantities of conidiophores and conidia within seed inoculated with AF36 either alone or in combination with an S strain isolate, but failure of conidiophores to form in seed inoculated with S strain isolates alone.

In no case did coinoculation with AF36 increase loss of locule weight over losses associated with inoculation of S strain isolates alone. Apparently, *A. flavus* AF36 does not have increased boll rot ability compared with the tested S strain isolates. Thus, ability to competitively exclude aflatoxin-producing isolates is not necessarily associated with increased boll rot ability. Indeed, AF36 had less boll rot ability than two S strain isolates (YV1-1 and PM 3) (Table 1) that it was effective at excluding.

There is interest in developing *A. flavus* AF36 as a biological control agent that can increase cottonseed safety by decreasing the aflatoxin content (7,8). The ability of isolate AF36 to inhibit sclerotial formation in developing cottonseed by highly aflatoxigenic isolates of the S strain may serve to reduce overwintering of S strain isolates and increase the long-term impact of AF36 on the aflatoxin-producing potential of fungal populations. Decreasing the incidence of sclerotia within infected cottonseed is a second mechanism by which AF36 may cause improved commodity safety.

## ACKNOWLEDGMENTS

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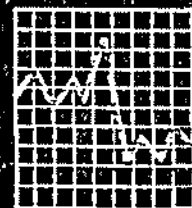
# Proceedings of the Third National IPM Symposium/Workshop

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## Broadening Support for 21st Century IPM

Edited by Sarah Lynch, Catherine Greene, and  
Carol Kramer-LeBlanc



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frequent rain showers that occur in spring weather, which is the time of maximum EFB infection. Fenarimol has shown "kickback activity" in that it controls fungal spore growth up to 48 hours after the spores have germinated and begun to infect plant tissue. This feature again proves to be valuable in Oregon during wet springtime conditions when growers cannot get into their orchards to spray immediately after a rain because of muddy or slippery conditions.

The percent control of EFB in the five years prior to 1991 has been estimated at 0 to 10 percent. The use of chlorothalonil through emergency exemptions in 1991 and beyond has increased the level of control to 50 percent. The addition of fenarimol is estimated by knowledgeable experts to increase control to greater than 80 percent.

IR-4 has been involved in the development of magnitude-of-residue data to support a Section 18 Specific Emergency Exemption and ultimately a Raw Agricultural Commodity 408 tolerance for Section 3 registrations of the use of iprodione (Rovral®) on apples for the control of *Alternaria* blotch. Iprodione application timing will be based on models. Two models are presently under evaluation. One model is based on a threshold of 65 percent of leaves with symptoms during the period of rapid disease increase (mid-June). The other model is based on accumulation of degree days and hours of leaf wetness. The models will be used to make a decision about the timing of the first fungicide application; subsequent applications will be made at 2- or 3-week intervals. Research has shown that where the first spray of iprodione (Rovral® 4F) was applied when recommended by the models, disease severity and defoliation were not significantly greater than in the preventive treatment where iprodione was applied on a 2-week schedule. The use of either model provided a savings of five fungicide sprays in each of the two orchards evaluated, thereby reducing the chemical load in the environment.

The fungicide metalaxyl has a very specific mode of action. Downy mildew fungi, of which there are many species and genera, have the ability to produce large numbers of spores that can be disseminated and cause new infections through many cycles within a single growing season. These

two factors make it highly likely that insensitive strains of downy mildew fungi will develop. Ciba Crop Protection has employed fungicide mixtures to reduce this potential. They have packaged metalaxyl with Mancozeb, Chlorothalonil, or copper fungicides to prevent the development of metalaxyl-insensitive strains of downy mildew. IR-4 has been involved in the development of magnitude-of-residue data to support Raw Agricultural Commodity 408 tolerances for Section 3 registrations of the use of metalaxyl plus copper on many crops for the control of downy mildew. These crops include: arrugula, bok choy, chinese cabbage, collards, kale, mustard greens, turnip, swiss chard, raspberry, grape, and papaya.

These three examples are only a few of the many ways that fungicides can be used in IPM/crop protection programs that enhance both food and environmental safety. IR-4 will continue to work cooperatively with growers, grower groups, state scientists, federal scientists, and registrants in obtaining clearances for fungicide uses that provide more optimal pest-management strategies.

**Displacement of Aflatoxin-Producing Fungi from Cottonseed, Peter J. Cotty, Agricultural Research Service, USDA**

There are no reliable and economic methods for preventing aflatoxin contamination of cottonseed, and no products are currently marketed to prevent preharvest contamination. Insect management, irrigation practices, harvest timing, planting date, and crop-handling procedures can be optimized to limit contamination. However, even after optimization, under severe environmental conditions, crops will frequently contain unacceptable levels of contamination. Controls must be effective during crop development and after crop maturation both in the field and in storage. Furthermore, most contamination occurs in damaged bolls; thus, controls must prevent contamination of plant parts compromised by either physiological stress or predation. Meeting these requirements is difficult for procedures that must prevent formation of the relatively rare, highly contaminated seeds that often contain the most contamination. A biopesticide that meets these requirements is being developed. This biopesticide uses naturally occurring atoxigenic

strains (do not produce aflatoxins) of *Aspergillus flavus* to competitively exclude aflatoxin-producing fungi and, in so doing, to prevent aflatoxin contamination. The product is expected to provide economic benefit to cotton producers in severely affected portions of Arizona. The IR-4 Project Biopesticide Program is facilitating the development of this product by assisting in the registration process.

Aflatoxins are toxic, carcinogenic chemicals that frequently occur in foods and feeds. Health concerns have led to regulatory limitations on the aflatoxin content of foods throughout most of the world (Stoloff, van Egmond, and Park 1991). The most toxic and highly regulated aflatoxin is B<sub>1</sub> (Park and Stoloff 1989; Stoloff, van Egmond, and Park 1991). The fungus *Aspergillus flavus* causes aflatoxin contamination of cottonseed. Contamination results in losses for producers, processors, and animal industries that depend on cottonseed for feed (Park and Stoloff 1989). Whole cottonseed and/or cottonseed products are an important dairy and cattle feed. Aflatoxins in cottonseed are transferred to milk in slightly modified form (Park and Stoloff 1989; Park and Stoloff 1989). U.S. regulations prohibit aflatoxin concentrations over 0.5 µg/kg in milk. Milk may be destroyed and entire operations temporarily shut down and quarantined in dairies producing milk tainted with unacceptable aflatoxin levels (Emmett 1989). To prevent unacceptable aflatoxin levels in milk, the regulatory threshold for aflatoxin B<sub>1</sub> in cottonseed fed to dairy cows is 20 µg/kg (Park, Lee, Price, and Pohlanö 1988; Park and Stoloff 1989). Aflatoxin contamination of cottonseed can be minimized by early harvest, prevention of insect damage, and proper storage (Cotty 1991a; Cotty 1991b). However, even under careful management, unacceptable aflatoxin levels may occur via either unpreventable insect damage to the developing crop (Cotty and Lee 1989) or exposure of the mature crop to moisture prior to harvest (Cotty 1992) or during storage (Russell and Lee 1985), handling, transportation, or even use (Cotty 1991a).

*Aspergillus flavus* populations are highly complex and are composed of strains that differ morphologically, physiologically, and genetically (Bayman and Cotty 1991; Bayman and Cotty

1993; Cotty 1989). Differences among strains in ability to produce aflatoxins is well known (Davis and Dicner 1983), and aflatoxin-producing ability is not correlated with strain ability to colonize and infect developing cotton bolls (Cotty 1989). These observations led to the suggestion that atoxigenic strains of *A. flavus* might be used to exclude toxigenic strains through competition during infection of developing crops, thereby preventing aflatoxin contamination (Cotty 1989; Cotty 1994). In both greenhouse and field experiments, wound inoculation of developing cotton bolls and corn ears simultaneously with toxigenic and atoxigenic strains led to reductions in aflatoxin contamination of the developing crop parts as compared with controls inoculated with only the toxigenic strains (Brown, Cotty, and Cleveland 1991; Cotty 1990). Atoxigenic strains are effective at preventing post-harvest aflatoxin contamination both when the crop is infected naturally in the field and when it is inoculated after harvest (Brown, Cotty, and Cleveland 1991). Thus, competitive exclusion of aflatoxin-producing strains of *A. flavus* with atoxigenic strains of the same fungal species may provide a single method for preventing aflatoxin accumulation throughout crop production and utilization (Cole and Cotty 1990; Cotty 1989; Cotty 1990; Cotty 1994).

In the United States, aflatoxin contamination of cottonseed is most consistent and severe in the irrigated western desert valleys, where contamination is often associated with pink bollworm damage (Cotty 1991a; Cotty and Lee 1989). Cottonseed produced in these valleys has a relatively high value per acre because of high cotton yields and high demand for cottonseed within the area. Contamination levels are highly variable within fields, plants, and even bolls (Cotty 1991a; Cotty and Lee 1989; Lee, Wall, Cotty, and Bayman 1990). Contamination is often associated with seed exhibiting bright green-yellow fluorescence (BGYF) on the linters under ultraviolet light (1). BGYF cottonseed are typically those infected by *A. flavus* through insect wounds. Results of greenhouse studies suggest atoxigenic strains reduce aflatoxin contamination by competitively excluding aflatoxin-producing strains from the crop (Brown, Cotty, and Cleveland 1991; Cotty 1990; Cotty and Bayman 1993). During seasons when aflatoxin contamination is

severe, *A. flavus* populations increase as the cotton crop is produced (Lee, Lee, and Russell 1986). For atoxigenic strains of *A. flavus* to be useful during crop production, they must be applied at a time and in a manner that allows them to compete successfully with aflatoxin-producing strains. In theory, application of an atoxigenic *A. flavus* strain early in the season should give the atoxigenic strain preferential exposure to the developing crop and thus the advantage in competing for crop resources during infection and during *A. flavus* population increases associated with cultivation (Robens and Richard 1992).

An aflatoxin-prevention technology based on atoxigenic strains of *Aspergillus flavus* is being developed for use in the region of Arizona with the most frequent and severe aflatoxin contamination of cottonseed. Strains are seeded into cotton fields at lay by (immediately prior to first bloom). The strains are applied to the soil surface under the crop canopy in the form of colonized sterile wheat seed. When the crop is subsequently irrigated, the atoxigenic strain uses the resources in the colonized wheat seed, sporulates, and disperses to the crop. Wheat seed colonized by atoxigenic strain *Aspergillus flavus* AF36 has been evaluated in small-scale test plots since 1989. Strain seeding caused large and significant changes in the *Aspergillus flavus* population on the crop and in the soil. Applications resulted in the applied atoxigenic strain becoming dominant in the field and aflatoxin-producing strains becoming less frequent. These changes in the *A. flavus* populations were associated with great reductions (75 percent to 99 percent) in aflatoxin contamination (Cotty 1991b). Further tests showed that atoxigenic strain applications have a long-term influence on *A. flavus* populations resident in agricultural fields, suggesting atoxigenic strain applications may have benefits over multiple seasons and that long-term, area-wide changes in the aflatoxin-producing potential of *A. flavus* populations may be achieved. Results of field plot tests indicate that atoxigenic-strain applications do not increase the amount of *A. flavus* on the crop at maturity and do not increase the percent of the cottonseed crop infected by *A. flavus*.

*Aspergillus flavus* typically becomes associated with crops in the field during crop development

and remains associated with the crop during harvest, storage, and processing. Thus, crop vulnerability to aflatoxin contamination remains until the crop is ultimately used. Similarly, atoxigenic strains seeded into agricultural fields prior to crop development will remain associated with the crop until use and may provide long-term postharvest protection from contamination. Atoxigenic strains applied both prior to harvest and after harvest have been shown to provide protection from aflatoxin contamination of corn (Brown, Cotty, and Cleveland 1991), even when toxigenic strains are associated with the crop prior to application.

Economics of aflatoxin contamination will probably dictate the regions in which atoxigenic strains are used. We hope to produce materials for atoxigenic strain applications for \$5.00 per acre or less. If treatments are 70-percent effective and an average of 40 percent to 70 percent of seed is above 20 ppb and the benefit of having aflatoxin-free seed is \$20 to \$40/ton, then growers will gain an average return above an initial \$5/acre investment of \$0.60/acre to \$14.60/acre. Economics may be improved by both long-term and cumulative benefits resulting from strain ability to remain in fields until the next crops are planted. Benefits may also arise from the applied atoxigenic strains remaining with the crop until use and thus preventing increased contamination during transit and in storage at dairies.

Just as dust does not stay in the field in which it is raised, fungi do not stay in the field to which they are applied. Thus, over time, applications may reduce contamination in an area as a whole, facilitating the development of either gin-wide or community-wide management programs. In areas where multiple crops are affected by contamination (i.e., corn, cotton, and peanuts), treatments to one crop may benefit all crops. The economics of applications in such areas may be complex.

Development of a product based on atoxigenic strains and sold as an agrochemical would probably be the simplest course to producing an aflatoxin-control product. However, there are currently no products available for preventing aflatoxin contamination during crop development. Thus, the potential market for such products is

unclear. Failure to demonstrate a reliable and ready market for atoxigenic-strain-based products has limited industrial involvement in their development. Alternatives to company development may include development of pest control districts. Advantages of such programs include tailoring the atoxigenic strains and formulations to specific regions, increased cost effectiveness, and development of mechanisms for funding the monitoring of fungal populations.

The next step in development and commercialization of atoxigenic strains is the performance of large-scale commercial tests. These tests will determine how to fit the technology into commercial practice and how to assess benefits of large-scale applications. Because atoxigenic strains are considered biopesticides, such evaluations require entry into the pesticide registration process and granting by the U.S. Environmental Protection Agency of an Experimental Use Permit and an Exemption from Tolerance. Interregional Research Project No. 4 is facilitating the further development of atoxigenic strains by assisting with the registration process. An application to treat a portion of the 1996 commercial cottonseed crop has been submitted.

Dead, weakened, and partially decayed plant tissues are readily available in agricultural environments, and it is not feasible to prevent the use of these resources by fungi. Thus, fungi grow as our crops are grown, and these fungi become associated with the edible portions of the crop. A level of control over which fungi become associated with crops may be provided by seeding select fungal strains into agricultural fields. This selection and seeding of fungal strains may reduce the vulnerability to aflatoxin contamination of all crops grown in a treated area.

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## AGRICULTURE, AFLATOXINS AND ASPERGILLUS

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## INTRODUCTION

Human activities affect both the size and structure of fungal populations. Construction, war, recreation, and agriculture disrupt large expanses of vegetation and soil; disruption causes redistribution of fungal propagules and makes nutrients available to fungi. Many fungi, including the aspergilli, exploit these human engineered resources. This results in the association of large fungal populations with various human activities, especially agriculture. When crops are grown or animals raised, fungi are also grown. From a human perspective, most fungi associated with cultivation increase inadvertently. Human activity, however, partly dictates which and how many fungi occur and the fungi, both directly and through fungal products, influence human activities, domestic animals, and even humans themselves.

During warm, dry periods, several of the aspergilli increase rapidly in association with crops. These include aspergilli in the *Aspergillus flavus* group. Prior to 1960, interest in the *A. flavus* group resulted both from the use of certain strains in processing of agricultural products in Europe and the Orient (Beuchat, 1978), and from the ability of some strains to parasitize insects. In the early 1960's fungi in the *A. flavus* group were implicated as the producers of aflatoxins ("*Aspergillus flavus* toxins"), the toxins which poisoned thousands of poultry, pigs and trout; in trout these factors were associated with liver cancer (Goldblatt and Stoloff, 1983). It soon became apparent that aflatoxins also occurred in the human diet and that aflatoxins could pass from feed to milk with only slight modification (Goldblatt and Stoloff, 1983). The most common aflatoxin, aflatoxin B<sub>1</sub>, was found to be a potent hepatocarcinogen in rats and trout; carcinomas were induced at rates below 1 $\mu$ gkg<sup>-1</sup> body weight (Robens and Richard, 1992). Aflatoxin content of foods and feeds was eventually regulated in many countries (Stoloff *et al.*, 1991). In some products, such as milk or infant foods, aflatoxin levels below 0.02  $\mu$ gkg<sup>-1</sup> are mandated. Thus, for many, the focus of interest in this diverse and important fungal group became the production of aflatoxins.

There clearly are interactions between agriculture, and both aflatoxins and the fungi in the *A. flavus* group. Some consequences of these interactions are obvious, others are virtually unexplored. The relationship of crop contamination cycles to the life strategies of

*A. flavus* group fungi is uncertain. The role agriculture plays in structuring *A. flavus* populations and their toxigenic potential is also uncertain. This chapter will address some aspects of the interactions of *A. flavus* with humans and human activities; it includes suggestions on how these interactions may be altered to reduce human exposure to aflatoxins and other detrimental fungal traits.

## INFLUENCES OF THE *ASPERGILLUS FLAVUS* GROUP

### Effects of Aflatoxins on Humans and Domestic Animals

Although aflatoxins are most often noted for ability to induce liver cancer at very low doses, they can cause several problems of economic importance during animal production. The presence of relatively high levels of aflatoxins in feeds can lead to animal death; rabbits, ducks and swine are particularly susceptible ( $LD_{50}$  = 0.30, 0.35, and 0.62 mg/kg<sup>1</sup>, respectively; Pier, 1992). However, at much lower concentrations, aflatoxins have other effects on domestic animals including immunosuppression and reduced productivity (Pier, 1992; Robens and Richard, 1992). Once consumed, aflatoxins are also readily converted to aflatoxin M<sub>1</sub>, which occurs in milk and can thus cause both human exposure and sickness in animal offspring (Pier, 1992; Robens and Richard, 1992).

**Incidence of Health Effects due to Contaminated Foods.** In many developed countries, regulations combined with both an enforcement policy and an abundant food supply can prevent exposure of human populations, in most cases, to significant aflatoxin ingestion (Stoloff *et al.*, 1991). However, in countries where either food is insufficient or regulations are not adequately enforced, routine ingestion of aflatoxins may occur (Hendrickse and Maxwell, 1989; Zarba, *et al.*, 1992). In populations with relatively high exposure, a role for aflatoxins as a risk factor for primary liver cancer in humans has repeatedly been suggested, but is still not clear (Robens and Richard, 1992). However, aflatoxins cause a variety of effects on animal development, the immune system and a variety of vital organs. Exposure to aflatoxins, particularly in staples (i.e. corn or peanuts) of people dependent upon relatively few nutrient sources, must be considered a serious detriment. The relationship between aflatoxins and kwashiorkor may be only one reflection of this detriment (Hendrickse and Maxwell, 1989).

**Effects of Aflatoxins on Agricultural Enterprise.** Controversies regarding the possible role of aflatoxins in primary liver cancer of humans are moot in the contemporary international marketplace. Brokers and producers of agricultural commodities have found aflatoxins increasingly costly as careful monitoring of aflatoxins limits the use and value of contaminated products (Cappuccio, 1989). Regulations in most developed countries and even many less developed countries restrict the import of contaminated foods and feeds (van Egmond, 1991; Stoloff *et al.*, 1991). Assessing the aflatoxin content of crops is a routine aspect of brokering and often a prerequisite of shipping. Contamination is highly variable and allowable concentrations are at such low levels (some below 1 µg/kg<sup>1</sup>), that analysis prior to shipping cannot always ensure acceptable levels upon receipt, even if no increases occur during transit (Horwitz *et al.*, 1993). This increases commodity costs and can decrease competitiveness of imported products. Regulations applied more rigorously to imported than domestic products or set at zero, where the limit of detection determines the enforcement level, can serve as barriers to trade which again increase the cost of products. These increased costs may be the primary effect of aflatoxins felt by most consumers in developed nations.

**Effects of Aflatoxins on Health of Agricultural Workers.** Labourers engaged in production and processing of commodities may be exposed to aflatoxins through inhalation

(Shorwell, 1991). Crops grown under conditions favouring aflatoxin contamination often become covered with large quantities of *A. flavus* propagules. Furthermore, air in areas where contaminated crops are produced may contain thousands of propagules per cubic meter (Lee *et al.*, 1986). These propagules, which are mostly conidia, remain associated with the crops through harvest and processing. Conidia contain large quantities of aflatoxins (over 100 mg/kg<sup>-1</sup> in some strains; Wicklow and Shorwell, 1982). Since most contamination occurs in damaged crop components, fines and dust generated during crop processing have much higher toxin contents than the crop as a whole (Lee *et al.*, 1983). The conidia, fines, and dust, may be inhaled and thus pose an avenue of exposure to aflatoxins; this exposure has been quantified in certain cases (Shorwell, 1991). Recently, occupational exposure to aflatoxins through the handling and processing of contaminated agricultural products has been associated with increased risk of both primary liver cancer and other cancers (Alavanja *et al.*, 1987; Olsen *et al.*, 1988).

#### ***Aspergillus flavus* group Fungi as Allergens and Animal Pathogens**

Several allergic and infective conditions of humans and certain other vertebrates are caused by *Aspergillus* species (Rinaldi, 1983; St. Georgiev, 1992; Wardlaw and Gedes, 1992). These include allergic bronchiopulmonary aspergillosis and invasive pulmonary aspergillosis. The most common cause of most of these conditions is *Aspergillus fumigatus* (Rinaldi, 1983; St. Georgiev, 1992; Wardlaw and Gedes, 1992). However, other aspergilli, including members of the *A. flavus* group, are also often implicated.

**Insect Pathogen.** During epidemics of aflatoxin contamination, high concentrations of *A. flavus* group propagules are associated with most objects resident in fields, including insects; thus insects may serve as vectors (Stephenson and Russell, 1974; Widstrom, 1979). *A. flavus* readily grows and multiplies on insect damaged crops, insect frass and on insects themselves both as dead debris and as parasitized hosts (Sussman, 1951, 1952; Stephenson and Russell, 1974; Goto *et al.*, 1988). Many insects typically carry *A. flavus* group isolates internally and many insects are hosts of at least certain strains (Stephenson and Russell, 1974; Widstrom, 1979; Goto *et al.*, 1988). Domesticated insects are included among the hosts of the *A. flavus* group. Domesticated insect diseases include Stonebrood, a rare disease of the honey bee which is of minor importance to bee keepers (Gilliam and Vandenberg, 1990) and koji kabi disease of cultivated silkworm larvae (Ohtomo *et al.*, 1975; Goto *et al.*, 1988).

#### **Benefits of *Aspergillus flavus* group Fungi**

**Industry.** Fungi in this group have had a long history in processing to increase product utility and value. *A. flavus* group strains are used to produce enzymes for food processing and other industrial uses and even to produce therapeutic products such as urate oxidase and lactoferrin (Chavaler *et al.*, 1992; van den Hondel *et al.*, 1992; Ward *et al.*, 1992). A variety of traditional fermented food products have been made with fungi in the *A. flavus* group for centuries (Beuchat, 1978).

**Ecological Benefits.** Although *A. flavus* group fungi are not commonly recognised as beneficial, these ubiquitous organisms become dominant members of the microflora under certain circumstances and exert multiple influences on both biota and environment. These fungi are important degraders of crop debris and may play roles in solubilising and recycling crop and soil nutrients (Ashworth *et al.*, 1969; Griffin and Garren, 1976). *A. flavus* can even degrade lignin (Beets and Dart, 1989). As insect pathogens, these fungi may serve to limit pest populations (Wadhvani and Srivastava, 1985) and have even been considered potential agents to replace chemical insecticides (Roberts and Yendol, 1971).

## Contamination Cycles

**Contaminated components.** *A. flavus* causes a variety of plant diseases typical of largely saprotrophic "weak" plant pathogens (Widstrom, 1992). These diseases include boll, ear, and pod rots which result in both decreased yield and reduced quality (Shurtleff, 1980; Watkins, 1981). However, crop infection by *A. flavus* takes on a different importance than infections for which concern might focus on yield and quality loss, or increased free fatty acids. Aflatoxins are compounds regulated in parts per billion; yet, these toxins occur in certain infections at concentrations over 100,000  $\mu\text{gkg}^{-1}$ . This situation causes high-toxin-containing components to greatly exceed in cost the value of the same components if not contaminated. Variability among components of crops in aflatoxin content is extreme (Figure 1). Most infected components contain low aflatoxin concentrations (below 50  $\mu\text{gkg}^{-1}$ ). However, a small percent contain very high toxin levels, at times exceeding 500,000  $\mu\text{gkg}^{-1}$  (Cucullu *et al.*, 1966; Schade *et al.*, 1975; Lee *et al.*, 1990; Steiner *et al.*, 1992). In many cases, elimination of highly contaminated components (over 1,000  $\mu\text{gkg}^{-1}$ ) would result in a commodity with an acceptable average aflatoxin content (Schade *et al.*, 1975; Steiner *et al.*, 1992).

Crop components damaged by wounding or severe stress are colonised and decayed by a variety of fungi. During hot and dry conditions, fungi in the *A. flavus* group out compete many colonising microbes and become the prominent fungi degrading damaged components. In most crops the majority of contamination occurs in damaged plant parts (Wilson *et al.*, 1977; Lee *et al.*, 1983; Cotty and Lee, 1989). Damaged seed can be sorted from high value crops for less profitable use such as production of vegetable oil. However, crushing contaminated seed to produce oil concentrates aflatoxins in the resulting meal which is used for feed. Such toxic meal caused the first recognised aflatoxin problems; peanut meal caused turkey X disease in England and cottonseed meal caused trout hepatocarcinoma in the United States (Goldblatt and Stoloff, 1983). Such meal must either be detoxified (i.e. through ammoniation) or put to non-feed use (Park *et al.*, 1988).

Geography determines frequency and severity. Geographic location greatly influences frequency of contamination. Many agricultural areas at low elevation and between the latitudes 35 N and 35 S have perennial risk of contamination. Countries in this zone (which include many countries with insufficient food supply) may view elimination of aflatoxins from the food supply differently than countries whose major agricultural lands lie out of this zone (i.e. developed countries in Europe and North America). Producers of contaminated products may base allowable levels of aflatoxins on toxicological data, whereas consumer nations which rarely produce contaminated products may base allowable levels at the lowest level detectable (Stoloff *et al.*, 1991).

Contamination cycles can be considered perennial, sporadic or infrequent based on locale and crop. In all three situations, populations of *A. flavus* are long term residents. However, populations in different areas differ in magnitude (Figure 2) (Griffen and Garren, 1974; Manabe *et al.*, 1978; Shearer *et al.*, 1992) and possibly in the distribution of both qualitative and quantitative traits (Manabe *et al.*, 1978; Cotty, 1992b). During periods not conducive to contamination, perennial areas (i.e. the desert valleys of Arizona; Lee *et al.*, 1986) support higher *A. flavus* populations than areas with infrequent contamination, i.e. midwest corn producing areas (Shearer *et al.*, 1992). Areas with sporadic contamination may have perennial contamination at low levels but, have less regular exposure to important predisposing factors such as hot, dry conditions, i.e. contamination of corn in certain areas of the southeastern United States (Widstrom, 1992) or insect pressure, i.e. pink bollworm pressure on cotton in western Arizona (Cotty and Lee, 1989). During periods conducive to contamination, a shift in the microflora occurs and aflatoxin producing fungi become dominant colonisers and decayers.

Processes through which crops become contaminated with aflatoxins are varied and complex (Diener *et al.*, 1987). However, certain generalities might be suggested. Contamination cycles may be divided into three phases: Prebloom, Crop Development, and Post Maturation (Figure 2).

**Prebloom.** Contamination does not occur in the field during the period after crop removal and prior to bloom. However, both the microflora and crop may become predisposed to contamination. During this phase: 1. propagules (conidia, sclerotia, colonised organic matter) are dispersed through cultivation, planting, pruning or other activities of animals (including man) or the environment; 2. *A. flavus* populations fluctuate, first decreasing after crop removal and then, if conditions are favourable, increasing on debris from current and

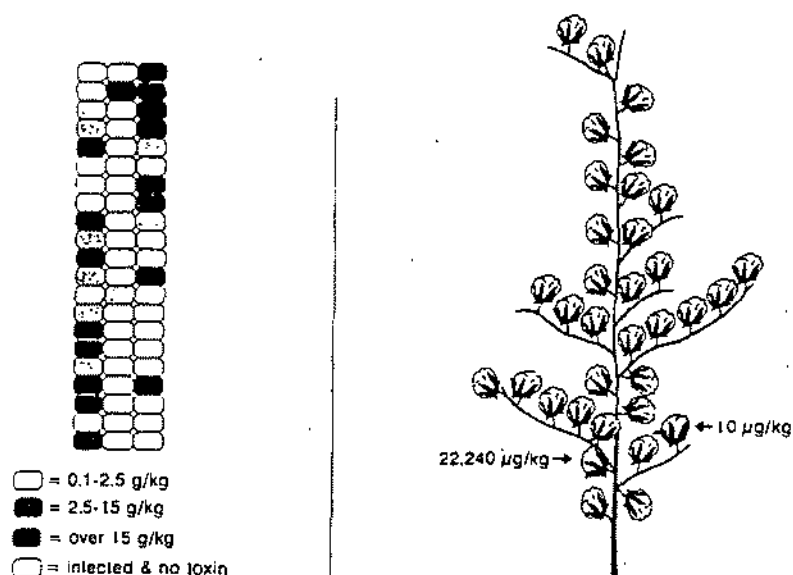


Figure 1. Distribution and aflatoxin content of maize kernels on an ear (drawn from data in Lee *et al.*, 1980) and bolls on a plant (Coty and Lee, 1990). Contamination is highly variable and not all infected seed becomes contaminated.

prior crops (Ashworth *et al.*, 1969; Griffen and Garren, 1974; Lee *et al.*, 1986) 3. The crop may become predisposed by long periods of drought or by luxuriant growth followed by drought (Cole *et al.*, 1982; Deiner *et al.*, 1987; Shearer *et al.*, 1992); 4. Overwintering insects emerge and develop.

**Crop Development.** From flowering to maturation, seeds and fruits are vulnerable to various perturbations. During this phase: 1. If conditions are hot and dry, populations of the *A. flavus* group, in canopy and soil, will outcompete many saprophytic microbes and increase in size. 2. High temperatures and/or drought stress may interfere with crop development and weaken plant defences making the crop more susceptible to infection and contamination (Jones *et al.*, 1981; Cole *et al.*, 1985; Wotton and Strange, 1987; Widstrom,

1992). 3. Wounding of fruits at middle to late stages of development can lead to portions of the crop with very high toxin levels (Lillehoj *et al.*, 1987; Cotty, 1989b). In several crops, most aflatoxin is formed during this phase and in certain locations crop predisposal to contamination can be attributed to specific wound types caused by specific insects. Examples are pink bollworm exit holes in cotton in the desert valleys of the western United States (Cotty and Lee, 1989), maize weevil damage in the southern United States (McMillian *et al.*, 1987), navel orange worm damage in nuts in the western United States (Schade *et al.*, 1975; Sommer *et al.*, 1986), and lesser corn stalk borer damage in peanuts in the southern United States (Lynch and Wilson, 1991). In some crops, components prevented from maturing due to stress or early harvest are particularly vulnerable to contamination (Cole *et al.*, 1985; Lynch and Wilson, 1991).

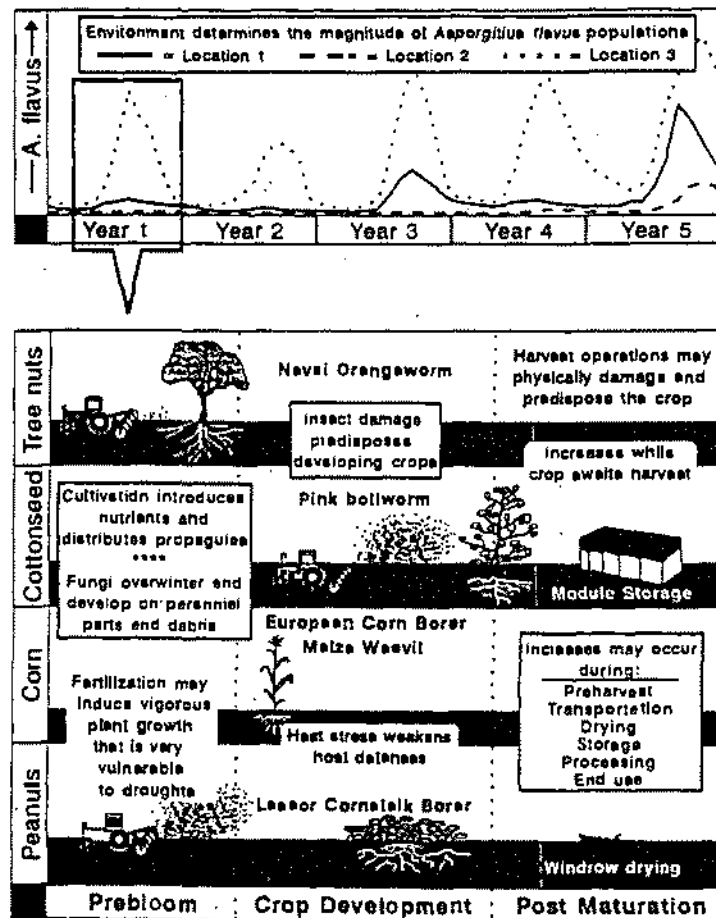


Figure 2. Contamination cycles can be divided into three phases. Local conditions determine both the extent of contamination and the magnitude of *A. flavus* populations associated with the crop. Boxed information applies to all crops.

**Post Maturation.** Most crops are susceptible to aflatoxin contamination at maturity and if the crop was grown in an area with perennial contamination or during a period conducive to contamination, the mature crop will be associated with large quantities of *A. flavus* group propagules. These propagules remain associated with the crop as it awaits harvest in the field, during harvest, field storage (i.e. peanuts in windrows, cotton in modules), shipment and processing, and even during storage by the end user. Exposure of the mature crop to periods of wetting and drying under warm conditions may lead to increased contamination. Aflatoxin concentrations are known to be dependent on environmental conditions and competing microflora (see Strain Isolation and Accumulation of Aflatoxins). Mature fruits and seeds are living organisms and factors which compromise seed health, such as wounding or stress, predispose these products to infection and contamination. Harvest operations can simultaneously damage crops and introduce *A. flavus* into wounds (Schroeder and Storey, 1976; Sommer *et al.*, 1986; Siriacha *et al.*, 1989). Insect activity after harvest can disperse aflatoxin-producing fungi and, by increasing host susceptibility, increase aflatoxin levels in a manner similar to insect damage during crop development (Dunkel, 1988). The same insect can affect contamination both prior to and after maturation (ie. the navel orange worm on pistachios).

Post maturation contamination dictates that each handler of the crop be responsible and minimize the potential for aflatoxin increases. Thus dairies which purchase feed with undetectable toxin must still store the feed properly or contaminated milk may occur. With indeterminate crops (e.g. cotton) crop development and post maturation phases may occur simultaneously and with all crops the prebloom and post maturation phases occur simultaneously, although at different locations.

Initially, the crop development phase was ignored because all contamination was thought to occur post harvest; recently, most research has been directed at contamination

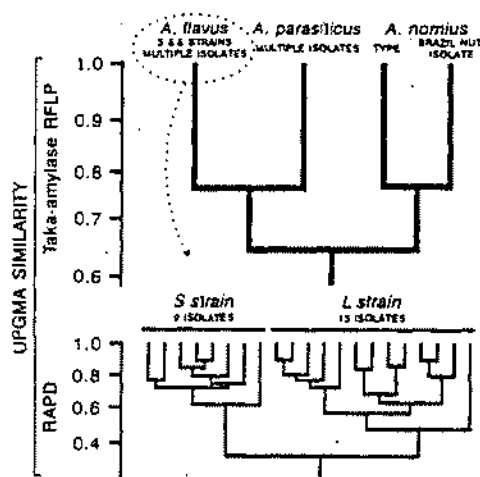


Figure 3. Phenograms of *A. flavus* group isolates. Taka-amylase data from Egel and Conly, (1992); RAPD data from Bayman and Conly, (1993).

before harvest, sometimes without distinguishing periods of active crop development from periods after maturation (Lillehoj *et al.*, 1976; Goldblatt and Stoloff, 1983). The contamination process can be divided many different ways besides those presented here. However, failure to segregate the contamination process into different phases may result in data that suggests no clear pattern and apparent contradictions. For example, in Arizona, most cottonseed contamination occurs during crop development in cottonbolls damaged by pink bollworms in the absence of rain (Cotty and Lee, 1989). Still, rain on a mature crop awaiting harvest can lead to significant contamination during post maturation, even if developing bolls were not damaged (Cotty, 1991). Similarly, a great fervour occurred about contamination of the midwest U.S. corn crop, in the field, during droughts of 1983 and 1988 (Kilman, 1989; Schmitt and Hurburgh, 1989; Shearer *et al.*, 1992). Yet, in Thailand, contamination typically occurs during the wet season, not during the dry season (Goto *et al.*, 1986). In Thailand's rainy season, contamination occurs during post maturation (Siriacha *et al.*, 1989); in the midwestern United States, it typically occurs during crop development (Lillehoj *et al.*, 1976).

## FUNGAL POPULATIONS

### Diversity

**Species of Aflatoxin-Producing Fungi.** There have been a variety of taxonomic schemes used to classify *A. flavus* group strains (Thom and Raper, 1945; Klich and Pitt, 1988; Samson and Frisvad, 1990). Each species represents an assortment of strains which behave as clonal organisms with the exception of occasional parasexuality between members of the same vegetative compatibility group (Papa, 1984, 1986). For the purposes of this discussion we will place all isolates within this group into four species *A. flavus*, *Aspergillus parasiticus*, *A. nomius*, and *Aspergillus tamarii*. Depending on interpretation, these species are supported by clustering algorithms based on DNA polymorphisms (Kurtzman *et al.*, 1987; Moody and Tyler, 1990a,b; Egel and Cotty, 1992; Bayman and Cotty, 1993). *A. tamarii* is of minor interest here because no isolates in this species produce aflatoxins. *A. tamarii* isolates apparently have some markedly different adaptations than the remainder of the group and *A. tamarii* is more distantly related to the other three species, than the three are to each other (Kurtzman *et al.*, 1987; Klich and Pitt, 1988). *Aspergillus oryzae* and *Aspergillus sojae* are apparently derived from *A. flavus* and *A. parasiticus*, respectively (Kurtzman *et al.*, 1986) and will be mentioned only in an industrial context. *A. nomius* was named after the genus of alkali bees from which several isolates were obtained (Kurtzman *et al.*, 1987). *A. nomius* comprises a group of strains that are distinct by both physiologic and molecular criteria (Kurtzman *et al.*, 1987; Bayman and Cotty, 1993). The name "*nomius*" may be misleading in associating this species predominantly with the alkali bee when isolates are known from several crops, including wheat (the type isolate) and peanuts (Hesseltine *et al.*, 1970).

**Diversity Within *Aspergillus flavus*.** Within each of the three aflatoxin producing species, there is a great deal of variability among isolates. It may be, that if we sought out all the unusual or atypical isolates within this group and examined them, we would find a continuum as suggested by Thom and Raper (1945). Indeed, based on polymorphisms in the Taka-amylase gene, we have found strains intermediate between *A. flavus* and *A. parasiticus* as well as *A. nomius* isolates almost as different from the *A. nomius* type strain as the *A. parasiticus* type from the *A. flavus* type (Egel and Cotty, 1992; see Brazil nut isolate in Figure 3). Variation among isolates is evident in genetic, physiological and morphological characters. Each of the above species is composed of at least several Vegetative Compatibility Groups (VCGs) and *A. flavus* is composed of many (Papa, 1986; Bayman and Cotty, 1991; P.J. Cotty, unpublished). Physiological and morphological traits are typically much more consistent within a VCG than within the species as a whole (Bayman and Cotty,

1993). Thus, a large portion of the variability perceived within *A. flavus* reflects divergence among VCGs. This divergence has resulted in consistent differences among VCGs in several characters, including enzyme production, plant virulence, sclerotial morphology, and other physiological traits (Cotty, 1989a; Cotty *et al.*, 1990b; Bayman and Cotty, 1993).

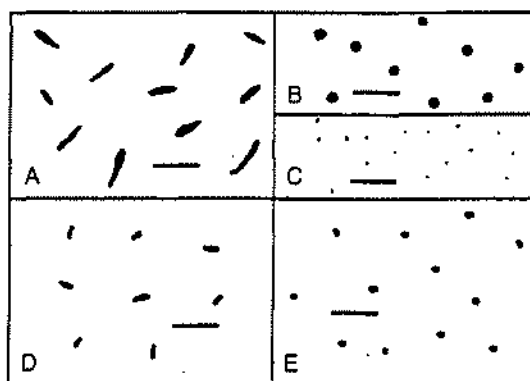


Figure 4. Silhouettes of sclerotia produced by aflatoxin producing isolates during 30 days growth at 32 °C on Czapek's agar; A is an unusual isolate of *A. nomius* from a Brazil nut; B is an L strain isolate of *A. flavus*; C is an S strain isolate of *A. flavus*; D is the type isolate of *A. nomius*; E is an isolate of *A. parasiticus*. All bars are 3 mm.

Variability in production of aflatoxins, especially among *A. flavus* isolates, has often been reported and discussed (Joffe, 1969; Davis and Deiner, 1983; Clevstrom and Ljunggren, 1985). *A. flavus* isolates may produce anywhere from no detectable aflatoxins ( $<1 \mu\text{gkg}^{-1}$ ) to over 1,000,000  $\mu\text{gkg}^{-1}$ . *A. parasiticus* and *A. nomius* produce B and G aflatoxins, and *A. parasiticus* produces aflatoxins far more consistently than *A. flavus* (Hesseltine *et al.*, 1970; Dorner *et al.*, 1984; Kurtzman *et al.*, 1987). Too few isolates of *A. nomius* have been examined to discern consistency. *A. flavus* is generally considered to produce only B aflatoxins (Samson and Frisvad, 1990); however, this observation is dependent on how the definition of *A. flavus* is restricted (Saito *et al.*, 1986; Klich and Pitt, 1988). Taxonomy aside, variability in toxin production, and other strain differences indicate divergence and possible differential adaptation. This variability can be a tool for discerning functions of variable traits (Cleveland and Cotty, 1991); it may further be used to develop a better understanding of the ecological niches to which strains are adapted.

On the basis of physiological and morphological criteria, *A. flavus* can be divided into two strains, S and L (Cotty, 1989a). Isolates in the S strain of *A. flavus* (actually a collection of strains which belong to numerous VCGs; Cotty, 1989a; Bayman and Cotty, 1993; Cotty *et al.*, 1990b) produce numerous small sclerotia and fewer conidia than other *A. flavus* isolates (Cotty, 1989a; Saito *et al.*, 1986). The L strain is composed of the so called "typical" isolates of *A. flavus* (Saito *et al.*, 1986) which produce larger and fewer sclerotia. Some key differences between the S and L strains are outlined in Table 1.

Table 1. Key characteristics of the S and L strains of *Aspergillus flavus*

Character	L strain	S strain	Reference <sup>1</sup>
Sclerotium size	Average > 300 mm	Average < 300mm	A, B
Production of aflatoxins	Variable, Zero to High	Consistent, High	A, B
Production of conidia	Heavy	Light	A, B
Production of sclerotia			
on potato dextrose agar	None to many, 10cm <sup>2</sup>	Many > 50cm <sup>2</sup>	A
on 5% V-8 juice	None to few, < 1cm <sup>2</sup>	Many 10cm <sup>2</sup> to 50cm <sup>2</sup>	A
Virulence to cotton	High	Low to High	A
Pectinase production	Consistent	Variable	C
Primary habitat	Aerial?	Soil?	

<sup>1</sup>References: A = Cotty, 1989a; B = Saito *et al.*, 1986; C = Cotty *et al.*, 1990b

**Importance of Infrequent Strains to Contamination.** The etiology of aflatoxin contamination, and the relationship of both the size and structure of *Aspergillus* populations to contamination is complicated by the importance of unusual strain types which occur at low frequency. Aflatoxin contamination is a peculiar and frustrating agricultural problem because less than 1% of the crop may be contaminated with levels high enough to make the average of the entire crop exceed allowable concentrations (Figure 1). During attribution of cause, infrequent but highly toxigenic strains may easily be overlooked or not identified as potential aflatoxin producers. Such may be the case with isolates belonging to the S strain of *A. flavus*. Due to colony and sclerotial appearance (Figure 4) S strain isolates may be passed over in favour of co-occurring "typical" or L strain isolates. Several visitors to our laboratory have been surprised at the identity of S strain isolates and have returned home to discover the occurrence of S strain isolates at their locale. In soils of several areas of the southern United States, the S strain incidence averages around 30% (Cotty, 1992b). On average S strain isolates produce much higher aflatoxin levels than L strain isolates, and also more sclerotia and fewer conidia (Saito *et al.*, 1986; Cotty, 1989a) (Table 1). Predominance of conidia of L strain isolates on mature crops may at times interfere with attribution of contamination to S strain isolates actually inciting the problem.

Another relatively infrequent aflatoxin-producing fungus is *A. nomius*. *A. nomius* isolates can produce large quantities of aflatoxins but may be misidentified as *A. parasiticus* which produces the same aflatoxins (both B and G) and roughened conidia (Hesseltine *et al.*, 1970; Kurtzman *et al.*, 1987). A case in point is an unusual *A. nomius* isolate from a store-bought brazil nut which contained 8,400 µg/kg<sup>1</sup> total aflatoxins (Figure 4). This isolate produces large quantities of aflatoxins and, based on polymorphisms in the taka-amylase gene, differs almost as much from other *A. nomius* isolates as *A. parasiticus* differs from *A. flavus* (Egel and Cotty, 1992) (Figure 3). This isolate is clearly unusual, but it incited significant contamination in the marketplace. Furthermore, such rare highly contaminated nuts are the primary source of contamination in brazil nuts (Steiner *et al.*, 1992).

**Diversity in Ecological Niches.** Fungi in the *A. flavus* group are broadly adapted to exploit many organic nutrients and to infect a variety of animal and plant hosts. Strains must adapt to compete in ecological niches which provide long term survival. Many strains with diverse adaptations clearly have some success in exploiting crop related resources. However, other niches, which may only support small fungal populations relative to crop associated niches, may have been occupied over long periods by certain strains. Differences among these "minor" niches may drive strain diversification. Similarly, stability of minor

niches may stabilise the character of minor strain types. Relatively stable minor niches may have greater long term importance than vast crop resources, because suitability and quantity of crop related resources oscillate widely in response to the environment, insect herbivory, changes in agronomic practice and the crop itself.

Wicklow (1982) showed that strains of the *A. flavus* group used as Koji moulds (moulds used to produce fermented foods) (Beuchat, 1978) germinate faster and have larger spores than wild strains of the species from which these moulds were probably domesticated; thus during domestication, the Koji moulds might have developed traits which favour rapid nutrient capture (and success during intraspecific competition) and lost traits which are not adaptive in the Koji environment, i.e. aflatoxin-producing ability (Wicklow, 1982). DNA relatedness among strains of *A. flavus* and *A. parasiticus* and their Koji mould equivalents, *A. oryzae* and *A. sojae*, suggest that the Koji moulds were indeed derived from the wild species (Kurtzman *et al.*, 1986; Egel and Cotty, 1992). However, attributing adaptive value to Koji traits is speculative in the absence of experimental data. Similarly, strain variability suggests multiple adaptations, but our assignment of specific functions to adaptations is largely speculative.

Strains of the *A. flavus* group may not only differ in host or nutrient use, but also in host/nutrient location and strategy to exploit resources. Members of this group are very common both in and above the soil. Although all *A. flavus* group strains contribute to the soil biota, certain strains may be better adapted to capture resources above the soil. Small sclerotia and reduced sporulation among S strain isolates may imply adaptation to infect and capture resources in the soil whereas relatively large sclerotia often facilitates aerial infection and nutrient capture (Garrett, 1960). S strain isolates may have diverged from other *A. flavus* strains through adaptations to the soil environment. There has been a general assumption that sclerotia of this group serve primarily to produce conidia after non-conductive periods (Wicklow and Donahue, 1984). Sclerotia of other fungi can germinate directly to infect hosts or capture resources (Colcy-Smith and Cooke, 1971); this may also be an important role for sclerotia in the *A. flavus* group, particularly for S strain isolates which appear to disperse via numerous, small sclerotia.

*A. parasiticus* has also been associated with the soil environment (Davis and Diener, 1983) because in certain locations (e.g. Georgia), it occurs more frequently on peanuts than on corn (Hill *et al.*, 1985). Although relatively few isolates have been compared, in most cases, *A. flavus* isolates are more invasive of crop tissues, even peanut tissues, than *A. parasiticus* (Calvert *et al.*, 1978; Zummo and Scott, 1990; Pin *et al.*, 1991). Furthermore, based on occurrence of G aflatoxins (produced by *A. parasiticus* and *A. niger*, but not *A. flavus*) *A. flavus* produces most contamination in peanuts (Hill *et al.*, 1985; Maeda, 1990). Conflicting observations on the association of *A. parasiticus* with peanuts may reflect fungal adaptations to soils or conditions in certain locales where peanuts are a major crop and lack of adaptation to other locales. This is supported by a low frequency of *A. parasiticus* in several agricultural areas and failure of introduced *A. parasiticus* strains to overwinter efficiently at certain locations (Davis and Diener, 1983; Zummo and Scott, 1990; Cotty, 1992b).

*A. flavus* strains produce large quantities of extracellular enzymes (van den Hondel *et al.*, 1992) which probably enhance their ability to utilise a broad assortment of organic resources. Enzyme polymorphisms have been used to suggest a role for specific enzymes (elastase and pectinase) in fungal virulence. Certain strains of *A. flavus* have reduced ability to rot cotton bolls and spread between cotton boll locules (Cotty 1989b). This reduced ability is associated with failure to produce a specific pectinase isoenzyme, P2C, both in culture and developing cottonbolls (Cleveland and Cotty, 1991; Brown *et al.*, 1992). In one population study, 50% of S strain isolates and no L strain isolates failed to produce P2C. This polymorphism suggests certain S strain clones are not dependent on efficient colonisation of plant hosts. S strain isolates might primarily exploit soil debris and/or insect hosis and thus

not require high pectinase. Even though not optimally adapted to exploit plants, pectinase P2C deficient strains do occur on the commercial crop and can cause significant contamination (Cotty, 1989a; Cotty *et al.*, 1990b). Therefore, specific adaptation to a crop is not required for strain contribution to contamination. Analogous to P2C variability is variability in production of elastase (an alkaline protease). All *A. flavus* strains isolated from patients suffering from invasive aspergillosis produced elastase whereas strains from other origins produced elastase less frequently (Rhodes *et al.*, 1988). Thus, a role for elastase in human pathogenesis has been suggested (Rhodes *et al.*, 1988), although this role is still controversial (Denning *et al.*, 1992). *A. flavus* is an opportunistic human pathogen and it's unlikely that *A. flavus* elastase evolved to permit infection of mammals. The ecological function of elastase is not clear; however, elastase production may be directed at exploitation of dead mammals or insects (Charnley, 1989; Malanthi and Chakraborty, 1991).

*A. flavus* has an intimate relationship with insects, particularly lepidopterans (Sussman, 1951, 1952). Excretion of large quantities of diverse enzymes, a characteristic of the *A. flavus* group (van den Hondel *et al.*, 1992), may facilitate mutualism as well as parasitism and saprophytism (Martin, 1992). Insect use of fungal excreted enzymes that degrade or detoxify plant products can drive development of fungal-insect mutualisms (Martin, 1992). The *A. flavus* elastase actively degrades multiple enzymes in alkaline environments (Rhodes *et al.*, 1990) and is relatively stable among other proteases (van den Hondel *et al.*, 1992; P.J. Cotty and J.E. Mellon, unpublished). Such activities might ameliorate the lepidopteran gut environment (ie. alkaline and high protease activity) (Martin, 1992) and permit strain establishment and retention. Similarly, aflatoxins may exert influence on insect immune systems (Charnley, 1989) permitting fungal strain retention. *A. flavus*-insect relations meet several predictions of mutualistic relations including fungal asexuality and lack of specificity (Martin, 1992). However, production of a potent insecticide and/or other virulence factors (Sussman, 1952; Ohtomo *et al.*, 1975; Drummond and Pinnock, 1990) within host tissues preclude full mutualism and allows a shift from avirulence to virulence. The associated host death may benefit both saprophytic insect exploitation and movement to plant resources (Bennett, 1981). Speculations about the nature of the relationship aside, diverse arthropods vector *A. flavus* group fungi, predispose crops to aflatoxin contamination and serve as both hosts and predators of many *A. flavus* group strains (Widstrom, 1979). In the latter two roles these animals may exert strong selective pressure on fungal strain character and the fungi may exert considerable pressure on insects (Rodriguez *et al.*, 1979; Wadhvani and Srivastava, 1985).

Specialisation of strains seems not to include pathogen-host specificity, or at least specificity has not been shown. Sussman (1951) showed diverse lepidopterans were infected by the same strain of *A. flavus* and isolates from one crop typically can infect and contaminate other distantly related crops (Schroeder and Hein, 1967; Brown *et al.*, 1991). Similarly, the life strategies of strains causing aspergillosis in poultry and humans are clearly not directed at specifically exploiting those hosts. Different crop associations of *A. parasiticus* and *A. flavus* strains may reflect either as yet undescribed adaptations to specific hosts or other differences in ecological adaptation and life strategy (Moss, 1991) (see above). Many adaptations in this group relate to aggressive saprophytism at elevated temperature and under relatively dry conditions. As pathogens, these fungi generally exploit wounded or stressed hosts and avoid taking on host defenses directly, although *A. flavus* does elicit plant defense mechanisms (e.g. enzyme production and phytoalexins) (Mellon, 1991, 1992). Still, healthy and non-compromised hosts (both plants and animals) can be infected (Barbesgaard *et al.*, 1992; Pitt *et al.*, 1992). Infection of healthy plant parts in the absence of symptoms may occur regularly, even if these infections do not include invasion of living host cells. Indeed, through serial isolations, systemic plant infections by *A. flavus* group strains have been observed in corn, peanut and cotton (Klich *et al.*, 1984; Pitt *et al.*, 1991; Mycock *et al.*, 1992).

## Influences of Agriculture on Fungal Populations

**Fungal Population Structure.** Populations of *A. flavus* group fungi are complex. All species within the group may occur on the same crop or in the same field (Schroeder and Boller, 1973; Davis and Diener, 1983; Cotty, 1992b). The greatest information on population structure is available for *A. flavus* which is composed of numerous vegetative compatibility groups (Papa, 1986; Bayman and Cotty, 1991) (Figure 5). Populations are complex at every level with multiple strains occupying gram quantities of soil and individual crop pieces. VCG composition of the population infecting a crop does not necessarily reflect the VCG composition of the population within the soil in which the crop is grown (Cotty, 1992b). Furthermore, during crop production new resources for *A. flavus* to exploit become available and population composition may change very rapidly (Bayman and Cotty, 1991; Cotty, 1991b, 1992b); apparently these fluxes in population composition are driven by establishment of relatively rare VCGs on newly available resources. There is little information on *A. flavus* group populations in the absence of agriculture and little information on fungal community responses to agricultural methods (Zak, 1992). However, it is clear that cultivation disturbs and homogenises the soil environment in which these fungi reside and in so doing must disperse conidia, sclerotia and colonised organic matter. At the same time both cultivation and crop development create immense resources for fungi to use. Although disturbance generally results in decreased species richness and heterogeneity, this sudden abundance of resources during environments favouring the *A. flavus* group may permit noncompetitive strain coexistence (Zak, 1992) and a temporary increase in the diversity of strains exploiting particular resources.

**Selection of Fungal Strains.** The *A. flavus* group is broadly distributed but, in the absence of crop cycles, *A. flavus* group populations are generally maintained at relatively low levels (Angle *et al.*, 1982; Shearer *et al.*, 1992) and in the absence of a conducive environment, *A. flavus* populations also maintain low levels on crop resources (Griffin and Garren, 1974; Shearer *et al.*, 1992). Thus, during conducive periods, there is a potential for crops to exert tremendous influence on strain growth and selection. Strains infecting crops are diverse in many characters including type and number of sclerotia, toxin producing ability, VCG, and even virulence to plants. This diversity among infecting strains suggests that agriculture does not aggressively select specific fungal types. However, a lack of requirement for aflatoxin production during crop infection and during fungal increases on crops (Cotty, 1989a) may permit disproportionate increases in atoxigenic strains (Bilgrami and Sinha, 1992). Furthermore, the importance of aerial dispersal to spread through a crop may cause the high sporulating L strain of *A. flavus* to outcompete the low sporulating S strain during secondary spread in the canopy. An as yet unknown specific strain-vector association could also permit strain advantage. Cropping process, crop types, geography, and/or climate may select certain strain types (Schroeder and Boller, 1973; Lafont and Lafont, 1977; Wicklow and Cole, 1982; Shearer *et al.*, 1992). However, multiple-year experiments with more rigorous design are needed to reliably establish such selection, if present. Studies should also utilise strain identification methods that are more specific than ability to produce either toxins or sclerotia. Vegetative compatibility analysis has been shown to be useful for monitoring the behaviour of specific strains over both time and space (Cotty, 1991b, 1992c) and we recently found differences among cotton producing areas in the proportion of *A. flavus* isolates belonging to the S strain (Cotty, 1992b).

Crops might exert different influences on populations by exposing strains to either different substrates or resistance factors. Crop components for which contamination is a concern (i.e. nuts or kernels) do not exert the only nor often the major influence on populations: other parts (i.e. leaves, stems, floral parts, cobs) may play a greater role in forming and maintaining the overall population (Zummo and Scott, 1990; Kumar and Mishra,

1991). Perennial crops (i.e. tree nuts) may maintain and select strains on perennial parts and long season crops (i.e. cotton) may provide longer periods of increase and shorter periods between crops than short season crops (i.e. corn). The nature and magnitude of plant debris and its successful survival between croppings may be an important determinant of population structure and magnitude (Jones, 1979; Zummo and Scou, 1990). *A. flavus* can colonise very large proportions of plant debris associated with crops and this debris can yield large quantities of conidia (Ashworth *et al.*, 1969; Stephenson and Russell, 1974). Variation among crops in insect microflora may also influence the composition of fungal populations. This phenomenon might occur due to differences in herbivory or variability among insect hosts in both life cycle and susceptibility to fungal strains.

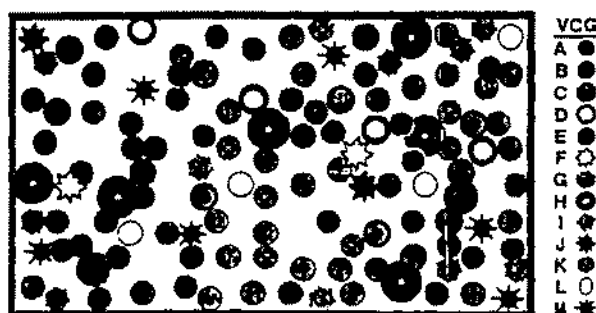


Figure 5. Diagrammatic representation of the vegetative compatibility group (VCG) composition of a single agricultural field. Marker frequency indicates the relative incidence of the represented VCG within the field. Marker shape does not reflect morphological differences among VCGs. Only VCGs that make up greater than two percent of the population are represented (Bayman and Cotty, 1991).

#### Adaptive Value and Ecological Significance of Aflatoxins

Through their effects on agriculture, aflatoxins prove to be "non-nutritional chemicals controlling the biology of other species in the environment," Torrsell's (1983) definition of secondary metabolites. A characteristic of secondary metabolites is a principally unknown function (Torrsell, 1983), however, consideration of the adaptive value of aflatoxins is appropriate when discussing interactions among aflatoxins, *Aspergillus*, and agriculture.

**Protection of Survival Structures.** High concentrations (3 mgkg<sup>-1</sup> to 132 mgkg<sup>-1</sup> aflatoxin B<sub>1</sub>) of aflatoxins occur in both conidia and sclerotia of aflatoxin-producing strains (Wicklow and Cole, 1982; Wicklow and Shewell, 1982; Cotty, 1988). The presence of aflatoxins in sclerotia has received the most attention because sclerotia are long term survival structures and aflatoxins are highly toxic to a variety of predators of fungi, especially insects (Wright *et al.*, 1982; Willens and Bullock, 1992). Sclerotia of *A. flavus* group fungi typically contain an extensive array of other toxic metabolites in addition to aflatoxins (Wicklow, 1990). Some of these metabolites are not found in other fungal structures and in combination with aflatoxins these toxins may form an elaborate chemical defense system directed at

protecting sclerotia from insect predation (Wicklow, 1990; Dowd, 1992). Indeed kojic acid, a metabolite of most *A. flavus* strains, can synergistically increase the toxicity of aflatoxin B<sub>1</sub> to caterpillars (Dowd, 1988).

Long term survival for *A. flavus* group propagules requires resistance to degradation by microorganisms during conditions (wet and/or cool) not conducive to successful competition by *A. flavus*. Bacteria are active under these conditions and, although aflatoxins are not very inhibitory to fungi, they do inhibit many bacteria at concentrations present in sclerotia (Burmeister and Hosseltine, 1966; Arai *et al.*, 1967; Angle and Wagner, 1981). Aflatoxins even inhibit certain known bacterial antagonists of *A. flavus* (Kimura and Hirano, 1988). However, pure aflatoxin B<sub>1</sub> is rapidly degraded in diverse soils (Angle, 1986), and thus to have long term effects, aflatoxins may themselves have to be shielded from decomposition, a condition possibly provided by the sclerotial rind (Willettts and Bullock, 1992).

*A. flavus* strains which produce sclerotia may either produce aflatoxins or not (Bennett *et al.*, 1979; Cotty, 1989a). Furthermore, sclerotia of the same strain with differing aflatoxin content can be produced by growing sclerotia on different substrates (Cotty, 1988). Sclerotia, from multiple sources, with different aflatoxin contents could be evaluated for longevity in field soil, resistance to microbial degradation, and insect predation. If aflatoxins contribute to the defense of sclerotia, some level of correlation between aflatoxin content and sclerotium resistance should occur.

**Association with Sclerotia.** A relationship between sclerotia and aflatoxins has been repeatedly suggested (Mehan and Chohan, 1973; Sanchis *et al.*, 1984). This is not a straightforward relationship because, *in vitro*, certain fungal strains produce aflatoxins but not sclerotia and vice versa (Bennett *et al.*, 1979). The situation is further complicated by attributing quantitative differences in toxin producing ability to the tendency of a strain to produce sclerotia (Mehan and Chohan, 1973; Sanchis *et al.*, 1984). These differences probably reflect differences among phylogenetically diverged groups which may be identified less ambiguously by sclerotial morphology (Cotty, 1989a). However, in strains that do produce both sclerotia and aflatoxins, there appears to be an interrelationship between regulation of aflatoxin biosynthesis and regulation of sclerotial morphogenesis (Cotty, 1988). This is suggested by: A) association of increases in aflatoxin production with inhibition of sclerotial maturation when cultures are exposed to either acidic pH or fungicides which inhibit ergosterol biosynthesis (Cotty, 1988; Bayman and Cotty, 1990); B) coincidence of sclerotial maturation with cessation of aflatoxin production (Cotty, 1988); C) high aflatoxin content of sclerotia (Wicklow and Cole, 1982; Wicklow and Shewell, 1982; Cotty, 1988); D) possible transport of aflatoxins from mycelia into sclerotia (Cotty, 1988; Bayman and Cotty, 1990). These observations suggest that sclerotium maturation is associated with a signal that terminates aflatoxin biosynthesis. Delays in sclerotial maturation may thus delay the termination signal and be associated with increased aflatoxin concentrations.

The interrelationship between sclerotial morphogenesis and aflatoxin biosynthesis is supported by recent advances in our understanding of the molecular biology of aflatoxin production. During characterisation of genes involved in aflatoxin biosynthesis, influences of specific genes on both biosynthesis and morphogenesis has been observed (Skory *et al.*, 1992). However, as Skory *et al.* (1992) point out, it is not clear whether this relationship is a direct influence of either aflatoxins or aflatoxin precursors on sclerotia or a regulatory association. The recent isolation of a putative regulatory element (*apa-2*) that influences both processes (Chang *et al.*, 1993) also corroborates the relationship. The suggestion of Skory *et al.* (1992), that aflatoxins themselves may serve a regulatory role during sclerotial development, is interesting in light of the ability of aflatoxin B<sub>1</sub> to directly bind DNA (Muench *et al.*, 1983); a regulatory role for aflatoxins in mature sclerotia is also possible.

**Accumulation of Aflatoxins in Substrates.** Aflatoxins are a concern in agriculture because large quantities can accumulate in certain plant materials. This accumulation may be a survival adaptation directed at either preventing ingestion of infested seed or inhibiting competition (Janzen, 1977; Bilgrami and Sinha, 1992). However, large quantities of aflatoxins are not accumulated in many plant parts in which the fungus increases and is maintained (Griffin and Garren, 1976; Takahashi *et al.*, 1986), and *A. flavus* is not very efficient at either degrading aflatoxins or converting them to use (Doyle and Marth, 1978). Many strains of *A. flavus* do not produce large quantities of aflatoxins (Davis and Deiner, 1983) and when grown with other microbes (which is common in nature) toxin production is greatly curtailed (see Strain Isolation and Accumulation of Aflatoxins). Indeed, it might be argued that most materials in which *A. flavus* grows and is maintained, are not contaminated with large quantities of aflatoxins. If accumulation of aflatoxins in plant substrates is a directed fungal strategy, it is a very inefficient one. Accumulation may be inadvertent, caused by interference with sclerotial morphogenesis (Cotty, 1988). Export of aflatoxins from producing cells (Shih and Marth, 1973) might be directed at creating accumulations in the sclerotial rind (Willems and Bullock, 1992). Accumulation of aflatoxins intracellularly and in dead cells of the sclerotial rind is testable by histological techniques.

**Microbial Interactions and Aflatoxin Biosynthesis.** Aflatoxin biosynthesis is readily inhibited by microbial competition. Many microbes interfere with aflatoxin production in culture (Kimura and Hirano, 1988; Roy and Chourasia, 1990) and in crops (Ashworth *et al.*, 1965; Ehrlich *et al.*, 1985). Even *A. flavus* and *A. parasiticus* strains and/or mutants which do not produce aflatoxins can interfere with aflatoxin production and/or crop contamination (Ehrlich, 1987; Cotty, 1990; Brown *et al.*, 1991). Interference apparently occurs through competitive exclusion (Cotty *et al.*, 1990a), production of interfering compounds (Shantha *et al.*, 1990) and/or competition for nutrients (Cotty *et al.*, 1990a). Isolation of aflatoxin-producing strains from interfering strains is thus prerequisite for accumulation of high aflatoxin concentrations in crops (Bullerman *et al.*, 1975; Roy and Chourasia, 1990). Isolation may occur spatially, as occurs in laboratory tests on sterilised substrates, or physiologically, usually when temperature or substrate moisture or composition favour dominance of *A. flavus*. Strain isolation may be one mechanism through which both wounding during crop development and high temperature favour very high toxin levels; rapid wound colonisation with aggressive invasion of developing tissues may permit such isolation. If isolation of toxigenic strains is inadequate, poor aflatoxin production will result even though conditions favour growth and reproduction of the inciting fungus (Bullerman *et al.*, 1975).

**Interactions with Hosts.** Aflatoxins are toxic and exert several physiologic effects on most hosts of the *A. flavus* group including plants, insects and mammals (Roberts and Yendol, 1971; McLean *et al.*, 1992; Robens and Richard, 1992). Aflatoxins may thus mediate pathogenesis either as a determinant of strain pathogenicity or by increasing strain virulence. Several lines of evidence suggest that this is, at least, not always the case. Even though very high toxin levels have been detected in plants (over 500 mg/kg<sup>-1</sup>) (Lee *et al.*, 1990) and insects (over 13 mg/kg<sup>-1</sup>) (Ohtomo *et al.*, 1975), *A. flavus* group isolates from insects, mammals, and plants may either produce aflatoxins or not. Furthermore, isolates which do not produce toxins retain ability to cause disease in the evaluated hosts (Cotty, 1989a; Drummond and Pinnock, 1990). In insects, aflatoxins may serve as virulence factors, increasing the rate at which infected insects die (Ohtomo *et al.*, 1975). However, the ability of *A. flavus* to infect and invade insects appears to be more dependent on enzymatic degradation of host proteins and cuticles (Sussman, 1952; Charnley, 1989).

Conservation of Aflatoxin producing ability. The *A. flavus* group is a mosaic of numerous strains delimited by a vegetative incompatibility system (Bayman and Cotty, 1991). These strains appear to evolve, at least in general, as clones (groups of identical organisms descended from a single common ancestor by mitosis) (King and Stansfield, 1985). These clones may move rapidly from rare to frequent depending on opportunity (Bayman and Cotty, 1991). The importance of a given trait to strain success may be viewed both by the diversity of strains expressing that trait and the frequency of the expressing strains. Consistent expression of a trait by diverse strains may indicate that trait is beneficial in multiple niches or that it has use in a broad ecological niche. The tendency to produce aflatoxins is highly variable within the overall *A. flavus* group. However, toxin production is more consistent among strains which are more closely related on the basis of morphological, physiological, genetic, or molecular characteristics (Dorner *et al.*, 1984; Saito *et al.*, 1986; Cotty, 1989a; Moody and Tyler, 1990a,b; Egel and Cotty, 1992; Bayman and Cotty, 1993). Thus, we can associate conservation of toxin production over evolutionary time with certain clusters of strains and loss of toxin producing ability with others. These observations may lead to new insights on potential adaptive values of aflatoxins as we learn more about the basic biology of the various clusters. Aflatoxigenicity is highly conserved among most wildtype strains of *A. parasiticus* and *A. flavus* strain S (Dorner *et al.*, 1984; Cotty, 1989a). Aflatoxin-producing ability is readily lost in culture and thus, conservation among field isolates of these evolutionarily diverged clusters implies a strong selective force causing retention of aflatoxin-producing ability. These clusters may share a common use for aflatoxins or may each have different uses. S strain isolates are more closely related to L strain isolates than to *A. parasiticus* (Egel and Cotty, 1992; Bayman and Cotty, 1993). Apparently divergence of the L and S strains is relatively recent compared to divergence of *A. flavus* and *A. parasiticus*. Unstable toxin production (Boller and Schroeder, 1974; Clevstrom and Ljunggren, 1985) and reduced toxin producing ability are characteristics of the L strain (Cotty, 1989a), and Bayman and Cotty (1993) found that low toxin producing strains within the L strain are more closely related to atoxigenic strains than to highly toxigenic strains. Thus, atoxigenicity apparently can be a multistep process in this group and for at least one of the ecological niches to which the L strain is adapted, aflatoxins do not confer an important advantage. Aflatoxins do not increase fungal virulence to crops (Coty, 1989a) and atoxigenic *A. flavus* strains have been associated with aerial crop parts (Bilgrami and Sinha, 1992); *A. parasiticus* (Davis and Deiner 1983) and S strain isolates have been associated with a soil habitat (see Diversity of Ecological Niches). Thus, the soil environment may favour conservation of toxin production and the aerial environment may not.

In addition to high aflatoxin production, reduced virulence to plants is also associated with the S strain of *A. flavus* (Coty, 1989a; Coty *et al.*, 1990b). Reduced virulence stems from failure to produce the most active *A. flavus* pectinase (Coty *et al.*, 1990b; Cleveland and Coty, 1991; Brown *et al.*, 1992). Low virulence, stemming from reduced ability to decay and colonise plant tissues (Brown *et al.*, 1991) may imply adaptation to a niche where such traits are not essential. Thus, high aflatoxin producing ability is associated with strains adapted to ecological niches where infection of crops is probably not essential.

Accumulation of large quantities of G aflatoxins by *A. niger*, *A. parasiticus*, and certain S strain isolates (Hesseltine *et al.*, 1970; Dorner *et al.*, 1984; Saito *et al.*, 1986; Kurtzman *et al.*, 1987), but not by other *A. flavus* isolates, provides an additional puzzle. Unique activities have not been associated with G aflatoxins. Therefore, it's difficult to envisage selective advantages conferred by retention of G aflatoxin producing ability. Production of G aflatoxins may merely reflect slight differences in pathway regulation (Bhainagar *et al.*, 1992) or retention of an ancestral trait.

## SELECTION OF ASPERGILLI ASSOCIATED WITH AGRICULTURE

### Strain Selection in the Past

*A. flavus* group populations have generally been altered by agriculture through disruption of habitat and introduction of nutrients. Production of crops under environmental conditions conferring a competitive advantage to these fungi permits their rapid increase on crop resources (Griffin and Garren, 1976; Lec *et al.*, 1986). After cropping, large quantities of crop remnants and debris are incorporated into field soil; additional remnants remaining after crop processing (from sorting, ginning, paring, shelling, etc.) are also often incorporated. This organic matter may both be superficially associated with large quantities of *A. flavus* group propagules and heavily colonised (Stephenson and Russell, 1974; Griffin and Garren, 1976). Under conditions particularly favourable to *A. flavus*, most organic debris incorporated into the soil can be colonised by *A. flavus* (Ashworth *et al.*, 1969). Strains associated with the crop and debris are diverse and generally not deliberately selected (Bayman and Cotty, 1991). Deliberate selection of specific strains with particular characters has been for production of enzymes for the European baking industry (Barbesgaard *et al.*, 1992) and for production of traditional fermentation products in the orient (Beuchat, 1978).

Fungal selection has reduced strain toxicity (Kurtzman *et al.*, 1986) and increased fungal traits associated with both product quality and efficient fermentation (Wicklow, 1982, 1990). Use of these fungi over centuries has inadvertently resulted in the release of large quantities of spores and colonised organic debris (Wicklow, 1990; Barbesgaard *et al.*, 1992). Such strain selection and release may have altered *A. flavus* populations in the vicinity of industries and may partly explain strain distribution (Manabe *et al.*, 1976). The validity of this speculation might be tested with recently developed techniques to characterise and compare structures of *A. flavus* populations (Bayman and Cotty, 1991, 1993).

### The Potential of Strain Selection

There are no methods for preventing aflatoxin contamination that are both reliable and economical. To fully protect crops from contamination, procedures must be active in the field under hot, dry conditions that are not very conducive to crop development but, often are near optimal for *A. flavus* group fungi. Controls must be effective during both the crop development and post-maturation phases of contamination cycles. The procedure must fit within agriculture's economic constraints and for worldwide use, must be effective under suboptimal storage conditions and with low technological input. Furthermore, because most contamination occurs in damaged seed (which for many crops either cannot be sorted out or must be used) controls must prevent contamination of plant parts compromised by either physiological stress or predation. These are difficult requirements for a procedure directed at preventing the relatively rare, highly contaminated seed.

A promising avenue of control, that may meet the above criteria, is the seeding of agricultural fields with atoxigenic *A. flavus* group strains in order to reduce toxigenicities of resident populations (Cotty 1991b, 1992a). *A. flavus* does not require aflatoxins to infect crops and production of large quantities of aflatoxins in crop parts does not increase either strain virulence or strain ability to colonise and utilise crop resources (Cotty, 1989a). This led to speculation that applied atoxigenic strains might outcompete toxigenic strains during crop infection and thereby reduce contamination (Cotty, 1989a; Cole and Cotty, 1990). Greenhouse and field experiments in which either developing cotton bolls or developing corn ears were wound inoculated with various strain combinations demonstrate the potential of atoxigenic strains to reduce contamination (80 to 90%) during crop development (Cotty, 1990; Brown *et al.*, 1991). Individual crop components are often coinfecting by multiple *A. flavus* strains and cottonbolls damaged by pink bollworms are infected by *A. flavus* strains in

multiple vegetative compatibility groups at least 50 to 80% of the time (Bayman and Cotty, 1991; P.J. Cotty, unpublished). Pink bollworm-damaged bolls contain the majority of aflatoxins in commercial fields (Cotty and Lee, 1989). Thus, the ability of atoxigenic strains to interfere with contamination in co-infected bolls may be of real practical value. Atoxigenic strains reduce contamination by both spatially excluding toxigenic strains and by competing for resources required for production of aflatoxins (Cotty *et al.* 1990a). However, not all atoxigenic strains are capable of reducing contamination during co-infection (Cotty, 1992a); thus strain optimisation in co-inoculation tests should be pre-requisite to field evaluation.

In theory, seeding fields with atoxigenic strains relatively early in crop development may permit seeded strains to compete with other resident strains for crop associated resources (Cole and Cotty, 1990). The seeded strains may thus increase in population size along with toxigenic strains when environmental conditions favour aflatoxin contamination (Cole and Cotty, 1990; Cotty, 1992a,c). At the same time, the atoxigenic strains may compete for infection sites. In special environmental control plots, Dörner *et al.* (1992) have demonstrated that *A. parasiticus* strains which accumulate specific aflatoxin precursors (i.e. a native strain that accumulates O-methylsterigmatocystin and a mutant that accumulates versicolorin-A) but not aflatoxins, can interfere with aflatoxin contamination when concentrated propagule suspensions are applied to developing peanuts. Applications resulted in long term (several years) fungal population changes and support the use of atoxigenic *A. flavus* group strains in preventing contamination of peanuts. The most comprehensive field tests, to date, have been performed on cotton grown in Yuma County, Arizona. This area has the most consistent aflatoxin contamination of cottonseed in the United States (Gardner *et al.*, 1974). An atoxigenic strain was seeded on colonised wheat seed (Cotty, 1992a) into a field of developing cotton, prior to crop flowering (Cotty, 1991b). The distribution of vegetative compatibility groups (VCGs) within this field had been determined in previous years, (Bayman and Cotty, 1990) and a strain in a rare VCG was seeded. Five months later the crop was harvested and the distribution of the applied strain on the crop was determined by mutating isolates to nitrate auxotrophy and assessing VCG. Strain seeding resulted in large and significant reductions in the aflatoxin content of the crop at maturity and aflatoxin content was inversely correlated with the incidence of the seeded VCG (Cotty, 1991b). Similar tests, performed in subsequent years, also demonstrate that atoxigenic strains applied early in crop development can partially competitively exclude toxigenic strains and thereby reduce contamination (Cotty, 1992c); this early strain application is associated with neither increased crop infection nor increased *A. flavus* populations on the crop at maturity.

The theoretical advantage of atoxigenic strains of *A. flavus* over other microorganisms that might be used to competitively exclude aflatoxin-producing strains is that atoxigenic strains are apparently adapted to similar environmental conditions as toxigenic strains. Other potential agents, such as bacteria (Kimura and Hirano, 1988; Bowen *et al.* 1992), may be inactive under the hot, dry conditions associated with aflatoxin contamination. The use of atoxigenic strains seeks to limit neither the amount of crop infection by the *A. flavus* group nor the quantity of these fungi associated with the crop. The procedure merely selects which fungi become associated with the crop. Thus, crop quality losses typically associated with fungal infection (i.e. increased free fatty acids) will not be ameliorated. Seeding atoxigenic strains might not result in increased crop infection because infection is more heavily dependent on host predisposition and the environment than on the number of propagules of *A. flavus*. Indeed in three years of tests on cotton, seeding has not resulted in increased infection rates (Cotty, 1992c). However, under certain circumstances with sufficiently low initial *A. flavus* levels and sufficiently high seeding rates, increased infection rates in treated crops might be expected. However, *A. flavus* typically decays predisposed crop components that, under different environmental conditions, would be infected by other microbes. Thus, these infections probably would not be of a magnitude to cause concern.

Populations of *A. flavus* increase on crops very rapidly under conditions favourable

to contamination. The ultimate magnitude of the *A. flavus* group is largely dependent on the available resources and the environment. Thus, even in areas with perennially low aflatoxin contamination, high *A. flavus* populations can rapidly develop during droughts (Shearer *et al.*, 1992); the composition of these rapidly increasing populations might be partially controlled by properly timed seeding.

*A. flavus* group fungi typically become associated with crops in the field during crop development and remain associated with the crop during harvest, storage and processing. Thus, seeding of atoxigenic strains into agricultural fields prior to crop development may provide postharvest protection from contamination by associating the harvested crop with high frequencies of atoxigenic strains. Atoxigenic strains applied both prior to harvest and after harvest have been shown to provide protection from aflatoxin contamination of corn (Brown *et al.*, 1991), even when toxigenic strains are associated with the crop prior to application.

Domestication of *A. flavus* group fungi for seeding into agricultural fields may cause some concern over the pathogenic potential of these fungi to humans (Pore *et al.*, 1970). Although limiting exposure of high risk individuals to aspergilli will reduce infection risk, particularly in hospitals, it might be argued that host predisposal is more important in determining disease incidence than exposure to fungal propagules (Wardlaw and Geddes, 1992; St. Georgiev, 1992; Rinaldi, 1983). In many agricultural industries and communities, workers and residents respire high concentrations of *Aspergillus* spores. Clearly such exposure is undesirable, but such respiration may occur without noticeable disease. This point is particularly clear for fungal strains used to produce koji and baking or brewing enzymes (Barbesgaard *et al.*, 1992). In these industries, generations of workers have been exposed to very high concentrations of spores throughout their working years with a very low incidence of disease (Barbesgaard *et al.*, 1992). Barbesgaard *et al.* (1992) argues for *A. oryzae* to be classified as "Generally Regarded As Safe" (GRAS), partially on this basis.

Seeding agricultural fields with select fungal isolates can result in *A. flavus* populations with altered composition, but without increased population size (Cotty, 1991b, Cotty 1992c). Thus, seeding may provide the opportunity to improve the overall safety of fungal populations by reducing human exposure to aflatoxins through both dietary and respiratory routes (see Effects of Aflatoxins on Humans and Domestic Animals). The frequency of fungal traits other than aflatoxin-producing ability might also be altered and, in so doing, fungal virulence to animals might be reduced or fungal sensitivity to therapeutic agents might be increased (Cotty and Egel, 1992). Other fungal traits detrimental to humans or human activities (i.e. allergenicity) might also be minimised and beneficial traits (e.g. ability to decay crop debris between plantings) might be maximised. The concept of fungal seeding also applies to fungi other than *A. flavus*, particularly to other aspergilli. *A. fumigatus*, a more potent animal pathogen than *A. flavus*, is a very frequent degrader of plant debris (Gandolla and Aragno, 1992). Extremely high concentrations of *A. fumigatus* spores may be associated with composting organic matter (Gandolla and Aragno, 1992). It may be possible to select strains of *A. fumigatus*, in a manner similar to *A. flavus*, in order to optimise both safety and decomposition.

This strategy of seeding fields with select strains of *A. flavus* has drawn repeated controversy and criticism based on the dangers of *A. flavus* populations (Wicklow, 1993; Kilman, 1993). However, the choice presented is not whether or not there will be fungi. The choice is whether we will determine, through deliberate selection, which strains make up the populations. Current agricultural practice does seed fields with very large quantities of organic matter colonised with *A. flavus* group fungi. This material is in the form of crop remnants, gin trash, corn cobs, etc. It is common practice to incorporate such materials into field soils. This differs from the seeding strategy suggested here in that seeded strains are not selected, the quantity of material incorporated is very large, and incorporation is not timed to give applied strains preferential exposure to the developing crop.

## Ecological Significance

The use of atoxigenic strains of *A. flavus* to control aflatoxin production has been hindered by a lack of information about fungal population biology. In many ways this field lags twenty or thirty years behind comparable studies on animals and plants (Burnett, 1983); the best-known fungus in this respect is probably *Neurospora crassa* (Perkins and Turner, 1988). Dispersal, change in population structure over time, and natural selection are poorly understood in fungal populations. This partly results from difficulty in tracking individuals (McDonald and Martinez, 1991). Interactions between conspecific genetic individuals have not been widely regarded until recently (Rayner, 1991). Furthermore, studies on one group of fungi have often turned out to have limited application to other groups. All these problems are complicated in fungi like the *A. flavus* group by tremendous reproductive and dispersal abilities, the lack of a known sexual stage, parasexuality (Papa, 1984), and mitotic chromosomal rearrangements (Keller *et al.*, 1992).

During the course of experiments discussed here, data has been collected on variation in many characters in many natural isolates. Areas have been sampled repeatedly over several years and known isolates have been introduced into fields and their survival and dispersal followed over the course of years; this has not been done with *Neurospora*. This body of data on how *A. flavus* genetic individuals survive, spread, and interact, may turn out to be as interesting as the biocontrol strategy it was designed to support.

One concept in sustainable agriculture is to "study the forest in order to farm like the forest" (Jackson and Piper, 1989). Understanding distribution, variation, and competition in fungal populations in nature and agriculture may lead to successful use of this principle.

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## Variability among Atoxigenic *Aspergillus flavus* Strains in Ability To Prevent Aflatoxin Contamination and Production of Aflatoxin Biosynthetic Pathway Enzymes

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Five strains of *Aspergillus flavus* lacking the ability to produce aflatoxins were examined in greenhouse tests for the ability to prevent a toxigenic strain from contaminating developing cottonseed with aflatoxins. All atoxigenic strains reduced contamination when inoculated into developing bolls 24 h prior to the toxigenic strain. However, only one strain, AF36, was highly effective when inoculated simultaneously with the toxigenic strain. All five strains were able to inhibit aflatoxin production by the toxigenic strain in liquid fermentation. Thus, *in vitro* activity did not predict the ability of an atoxigenic strain to prevent contamination of developing bolls. Therefore, strain selection for competitive exclusion to prevent aflatoxin contamination should include evaluation of efficacy in developing crops prior to field release. Atoxigenic strains were also characterized by the ability to convert several aflatoxin precursors into aflatoxin B<sub>1</sub>. Four atoxigenic strains failed to convert any of the aflatoxin biosynthetic precursors to aflatoxins. However, the strain (AF36) most effective in preventing aflatoxin contamination in developing bolls converted all tested precursors into aflatoxin B<sub>1</sub>, indicating that this strain made enzymes in the aflatoxin biosynthetic pathway.

Aflatoxins are toxic, carcinogenic compounds produced by *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius* (17, 22). Aflatoxin contamination of various commodities can occur as a result of crop infection by one of these fungi. Animal and human health concerns about aflatoxin-tainted commodities have resulted in stringent regulations worldwide on aflatoxin content; these regulations on aflatoxin contamination have a significant international economic impact (26). Aflatoxin B<sub>1</sub> originates from a polyketide precursor according to the following scheme (4, 5): polyketide precursor → norsolorinic acid → averantin → averufanin → 1'-hydroxyversicolorone → versiconal hemiacetal acetate → versicolorin B → versicolorin A → denterthylsterigmatocystin → sterigmatocystin → O-methylsterigmatocystin → aflatoxin B<sub>1</sub>.

Most contamination of corn, cottonseed, and tree nuts is caused by *A. flavus* (17). The incidence of contamination is largely determined by the environment, with preharvest contamination being favored under hot, dry conditions (17, 28). The lack of reliable and practical methods to prevent contamination when environmental conditions are most conducive to *A. flavus* (13, 28) has resulted in a variety of new technologies (8). One such technology is the use of atoxigenic strains of the causal agent (i.e., strains which do not produce aflatoxins) to prevent contamination through competitive exclusion of toxigenic strains during infection (6, 10, 12). Cotton has been used as a model crop for the development of the atoxigenic strain strategy because (i) there exists an easily manipulated greenhouse disease model for cotton (24), (ii) aflatoxin contamination of cottonseed is an important economic problem (13, 17), and (iii) cottonseed is grown for feed and not food and may thus provide an easier target for regulatory approval of atoxigenic strain use.

Although atoxigenic strains are known to vary in the ability to prevent contamination of cottonseed by toxigenic strains, all seven strains examined thus far showed some effect (12). Little else, however, is known about atoxigenic strain characteristics and the relation of various characteristics to strain efficacy.

In this study, we compared the atoxigenic strain with the greatest known efficacy (isolate AF36 [12]) with other previously identified, frequently cited (10, 20, 23) atoxigenic strains with respect to the ability to prevent toxigenesis both in liquid fermentation and during infection of developing cotton bolls. We have also characterized the atoxigenic strains according to the ability to produce enzymatic activities in the aflatoxin biosynthetic pathway. Relationships among enzymatic activities, phenotype stability, and strain efficacy are discussed.

### MATERIALS AND METHODS

**Organisms and media.** The origins and characteristics of the *A. flavus* strains used in this study have been described (11, 20). Strain AF36 was isolated by the author (11) and shown to be very effective at reducing aflatoxin contamination of developing cotton bolls (12); strains NRRL-5918, NRRL-5565, NRRL-5917, and NRRL-1957 were supplied by S. W. Peterson of the National Center for Agricultural Utilization Research, Peoria, Ill. Isolates were maintained and stored as previously described (11). Inocula for experiments consisted of suspensions of spores from 7-day-old cultures grown on 5% V-8 vegetable juice-2% agar, pH 5.2, at 30°C.

**Greenhouse tests.** Greenhouse tests to assess strain efficacy were performed as previously described (12). Twenty-eight, 10- to 32-day-old bolls were each inoculated in a single locule through a simulated pink bollworm exit hole made with a cork borer (3-mm diameter). Each wound was inoculated with a 10-μl aliquot of a spore suspension containing approximately 2,000 spores; bolls inoculated with two strains received a 10-μl aliquot of each strain. Bolls were inoculated either with toxigenic strain AF13 alone, with AF13 and an atoxigenic strain (either AF36, NRRL-5918, NRRL-5565, NRRL-5917,

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TABLE 1. Effects of five atoxigenic strains of *A. flavus* on the ability of toxigenic strain AF13 to contaminate developing cottonseed with aflatoxin B<sub>1</sub>.

Atoxigenic strain	Test 1		Test 2	
	Concn of aflatoxin B <sub>1</sub> (µg/g) <sup>a</sup>	% Change <sup>b</sup>	Concn of aflatoxin B <sub>1</sub> (µg/g) <sup>a</sup>	% Change <sup>b</sup>
AF36	45 C	-94	5 B	-86
NRRL-5565	230 CB	-71	38 A	NS
NRRL-5918	327 CB	-57	59 A	NS
NRRL-5917	427 CBA	NS	30 A	NS
NRRL-1957	553 BA	NS	86 A	NS
None <sup>c</sup>	769 A		37 A	

<sup>a</sup> Values are averages of five replicates in test 1 and six replicates in test 2. Values followed by the same letter are not significantly different by Fisher's protected least significant difference test.

<sup>b</sup> Percent difference in aflatoxin content of bolls inoculated with both toxigenic and atoxigenic strains and bolls inoculated with the toxigenic strain alone. NS, change not statistically significant ( $P = 0.05$ ).

<sup>c</sup> Plants were inoculated with the toxigenic strain AF13 alone.

or NRRL-1957) simultaneously, or with an atoxigenic strain first and then AF13 after 24 h. Randomized complete block designs were used, and experiments were performed at least twice.

At maturity (3 weeks after inoculation), bolls were harvested, dried at 60°C for 3 days, and kept at room temperature in plastic bags containing silica gel desiccant until analyzed for aflatoxin content. Aflatoxins were extracted by the method of the Association of Official Analytical Chemists (33) as previously modified (12). Briefly, intact bolls were pulverized and extracted with an 85% aqueous-acetone solution. The extract was purified, concentrated, and applied adjacent to aflatoxin standards on thin-layer chromatography plates. After development, the quantity of aflatoxin B<sub>1</sub> was measured with a densitometer with fluorescence capabilities (33).

In vitro tests and enzyme assays. Erlenmeyer flasks (250 ml) containing 70 ml of the defined growth medium of Adye and Mateles (1) were inoculated with approximately 5,000 spores of either an atoxigenic or a toxigenic strain separately or in combination. Flasks were incubated on a rotary shaker at 30°C and 150 rpm for 5 days, at which time 70 ml of acetone was added to each flask to kill the culture and solubilize secreted and cellular aflatoxin. After filtration, equal volumes of water were added to the extracts, the resulting solutions were each extracted twice with 25 ml of methylene chloride, and the extracts were combined and evaporated to dryness. The aflatoxin B<sub>1</sub> content of the extracts was determined by standard thin-layer chromatography procedures as described above.

Enzyme activities were determined by adding known quan-

ties of aflatoxin B<sub>1</sub> precursors to fungal cultures and measuring conversion of these precursors to aflatoxin B<sub>1</sub> as previously described (5, 25). Mycelia (1 g) of either AF36 or NRRL-5918 from 3-day-old cultures were transferred to 10 ml of low-sugar replacement medium containing either 2.0 µg of norsolorinic acid, 2.0 µg of averantin, 2.0 µg of averufanin, 1.0 µg of sterigmatocystin, or 0.6 µg of *O*-methylsterigmatocystin. After 6 h of incubation at 150 rpm and 37°C, metabolites were extracted and analyzed for aflatoxins. Precursor standards were chromatographed on the same plates as extracts to establish the presence or absence of spiked precursors.

## RESULTS

Developing cotton bolls inoculated simultaneously with both atoxigenic strain AF36 and toxigenic strain AF13 contained significantly less aflatoxin B<sub>1</sub> at maturity than bolls inoculated with AF13 alone (Table 1). During the present study, strain AF36 was the only consistently effective atoxigenic strain evaluated. Over the past 5 years we have evaluated AF36 in several similar tests for various purposes. All these tests involved at least two treatments: (i) bolls were inoculated with a toxigenic strain alone and (ii) bolls were inoculated simultaneously both with the same toxigenic strain as in treatment i and with AF36. In each of these 16 similar greenhouse tests, contamination by a toxigenic strain was significantly ( $P = 0.05$  by Fisher's least significant difference test) reduced by AF36 (an average reduction of 95.3%, with a standard deviation of 5.5%). Two atoxigenic strains (NRRL-5917 and NRRL-1957) were consistently ineffective at reducing contamination when simultaneously inoculated with toxigenic strain AF13 (Table 1), whereas two other strains (NRRL-5918 and NRRL-5565) were effective in only one test. Over the past 3 years, strain NRRL-5918 was further evaluated in an additional three similar greenhouse tests in which it was not effective. In liquid fermentations, however, atoxigenic strain NRRL-5918 greatly reduced toxin production by toxigenic strain AF13 (Table 2). This outcome held for the single test in which all five atoxigenic strains were tested and in both tests in which strains AF36 and NRRL-5918 were tested.

When developing cotton bolls were inoculated first with an atoxigenic strain and then 24 h later with a toxigenic strain, all the atoxigenic strains were effective at reducing the toxin content of seed at maturity compared with bolls inoculated with a toxigenic strain alone. Usually, bolls treated with an atoxigenic strain 24 h prior to treatment with a toxigenic strain contained no detectable toxin at maturity (Table 2).

Two atoxigenic strains were characterized by the ability to remove aflatoxin B<sub>1</sub> precursors from spiked cultures and convert these precursors to aflatoxin B<sub>1</sub>. Strain AF36 removed

TABLE 2. Influence of two atoxigenic strains of *A. flavus* on toxin production by toxigenic strain AF13 in culture and in developing cotton bolls.

Atoxigenic strain	Simultaneous inoculation				Plus (24 h) inoculation of bolls with atoxigenic strain	
	In culture		In cotton bolls			
	Concn of aflatoxin B <sub>1</sub> (µg/g) <sup>a</sup>	% Change <sup>b</sup>	Concn of aflatoxin B <sub>1</sub> (µg/g) <sup>a</sup>	% Change <sup>b</sup>	Concn of aflatoxin B <sub>1</sub> (µg/g) <sup>a</sup>	% Change <sup>b</sup>
AF36	7 B	-97	5 B	-98	11 B	-100
NRRL-5918	14 B	-98	384 A	+24	11 B	-100
None	210 A		309 A		769 A	

<sup>a</sup> Values are averages of four replicates. Values followed by the same letter in the same column are not significantly different by Fisher's protected least significant difference test. Flasks and cotton bolls inoculated with either NRRL-5918 or AF36 alone contained no detectable levels of aflatoxin B<sub>1</sub> (limit of detection, 111 ng/g).

<sup>b</sup> Percent difference in aflatoxin content between treatments with the toxigenic strain alone and treatments with both toxigenic and atoxigenic strains.

TABLE 3. Conversion of aflatoxin precursors to aflatoxins by 3-day-old cultures of *A. flavus* AF36

Precursor <sup>a</sup>	Amt of precursor ( $\mu$ g)	Amt of aflatoxin B <sub>1</sub> ( $\mu$ g)	Amt of aflatoxin B <sub>2</sub> ( $\mu$ g)	% Conversion <sup>b</sup>
None		ND <sup>c</sup>	ND	ND
Narsutorinic acid	2.0	0.24	0.03	12
Averantin	2.0	0.34	0.05	17
Averufanin	2.0	0.41	0.12	21
Sterigmatocystin	1.0	0.45	ND	45
O-Methylsterigmatocystin	0.6	0.32	ND	58

<sup>a</sup> Each precursor was fed in 10  $\mu$ l of acetone to 1 g of 3-day-old fungal mycelia in liquid medium replacement medium. After 6 h of incubation at 37°C and with constant shaking at 180 rpm, metabolites were extracted and analyzed for aflatoxins. No conversion was detected with strain NRRL-5918.

<sup>b</sup> Efficiency of conversion of metabolites to aflatoxin B<sub>1</sub>.

<sup>c</sup> ND, none detected (limit of detection, 0.01 ng).

all tested precursors from cultures and converted these to aflatoxin B<sub>1</sub>. Conversion efficiency increased with precursor closeness to aflatoxin B<sub>1</sub> in the aflatoxin biosynthetic pathway (Table 3). Strain NRRL-5918 did not remove any tested precursor from cultures and failed to produce aflatoxin B<sub>1</sub> in all spiked cultures. When no conversion of an introduced precursor was observed, greater than 70% of the precursor was recovered.

### DISCUSSION

Application of atoxigenic strains of *A. flavus* to agricultural fields and crops has been suggested as a potential method for preventing aflatoxin contamination (11, 15, 16). In theory, the applied atoxigenic strains will lower the potential for aflatoxin contamination by competing with aflatoxin-producing strains (10, 16). To date, field evaluation of this concept has been limited. Propagule suspensions of *A. parasiticus* strains which do not produce aflatoxins have been applied to peanuts in environmental control plots in Georgia (18), and autoclaved wheat seed colonized by an atoxigenic strain of *A. flavus* has been applied to cotton grown in field plots in Arizona (14, 16). In those studies, strain applications were associated with both fungal population changes and reductions in the quantity of aflatoxins contaminating the crop at maturity. In greenhouse and field tests, certain atoxigenic strains of *A. flavus* interfere with aflatoxin contamination of developing crops when these crops are inoculated simultaneously with both toxigenic and atoxigenic strains (6, 12). Cotton bolls naturally infected in agricultural fields become infected with multiple *A. flavus* strains at high rates (more than 50% of bolls were infected by multiple strains in one study) (2), and therefore the ability to interfere with contamination during coinfection might be of practical importance. The results reported here indicate that not all atoxigenic strains are effective at reducing contamination under these conditions. Efficacy during coinfection should be considered an important criterion when selecting strains for use in preventing aflatoxin contamination in commercial fields.

The results suggest that atoxigenic strains which fail to produce certain enzymes in the aflatoxin biosynthesis pathway (e.g., NRRL-5918) may not be more likely to reduce contamination by toxigenic strains than atoxigenic strains which do produce these enzymes. Indeed, strain AF36, which produced many of the enzymatic activities present in the pathway but did not produce aflatoxins, was the most effective atoxigenic strain at reducing contamination in the present study.

All four atoxigenic strains which lacked the ability to inhibit aflatoxin contamination of cottonseed when inoculated simultaneously with toxigenic strain AF13 did interfere with con-

tamination when inoculated 24 h before the toxigenic strain. These strains may thus be useful in aflatoxin control strategies seeking to competitively exclude toxigenic strains prior to crop infection, providing that strain displacement is very efficient. However, because of poor competitive ability, atoxigenic strains may fail to prevent aflatoxin production by a toxigenic strain during coinfection of developing crops.

Atoxigenic strain AF36 significantly reduced aflatoxin contamination of developing cottonseed in all tests. However, in one test, even though the aflatoxin content of the seed at maturity was reduced by 94%, the seed still contained 45  $\mu$ g of aflatoxin B<sub>1</sub> per g (Table 1). Thus, crops exposed to conditions highly conducive to aflatoxin contamination may contain unacceptable contamination levels even when effective doses of atoxigenic strains are applied. However, in most cases, a 90% reduction in contamination will result in a commercially useful cottonseed crop.

Strain NRRL-5918 interfered with aflatoxin production by toxigenic strain AF13 in liquid fermentation but not during infection of developing cotton bolls. Similarly, anthraquinone-accumulating mutants of *A. parasiticus* (19), non-aflatoxin-producing species of the *A. flavus* group (32, 34), and many other fungi (30) interfere with aflatoxin production in culture. The failure of NRRL-5918 to inhibit contamination during coinfection of developing cotton bolls indicates that in vitro interference with aflatoxin biosynthesis is not necessarily related to the ability to inhibit in vivo. Indeed, these results suggest that the mechanism of in vitro inhibition of aflatoxin biosynthesis may differ from that of in vivo inhibition.

Certain atoxigenic strains of *A. flavus* are known to be unstable and to convert to a highly toxigenic phenotype (9, 31). The stability of the aflatoxin-producing phenotype may be an important consideration in selecting strains for use in strategies to prevent aflatoxin contamination through intraspecific competition (6, 10). Neither phenotype described here can be considered more stable on the basis of current information, and each might result from a single mutation.

Strains NRRL-1957, NRRL-5565, NRRL-5917, and NRRL-5918 were previously shown to lack the ability to convert O-methylsterigmatocystin and sterigmatocystin to aflatoxin B<sub>1</sub> (23). The results presented here confirm those results. However, in the same report, Lee (23) suggested that production of aflatoxin biosynthesis enzymes by an atoxigenic strain is characteristic only of atoxigenic strains generated in the laboratory and that such enzyme-producing strains are not stable and may convert to a toxigenic form on introduction to a crop. Lee further suggested that these converted strains might cause a net increase in aflatoxin contamination. The results of the present study show that this is not the case. Strain AF36, which produces aflatoxin biosynthetic enzymes, was isolated from an agricultural field and consistently reduced contamination of developing cottonseed by toxigenic strains. Moreover, AF36 has been phenotypically stable through five serial single conidium transfers and in numerous mass transfers in our laboratory (data not shown).

The mechanisms of atoxigenicity of all five atoxigenic strains remain unknown. None of the examined strains accumulate large quantities of either anthraquinone or xanthone precursors of aflatoxins, as do certain atoxigenic strains of *A. parasiticus* (3). This is expected because although atoxigenic *A. flavus* strains are much more common than atoxigenic *A. parasiticus* strains, naturally occurring precursor-accumulating strains of *A. flavus* have not been described (3). Genes affecting aflatoxin biosynthesis occur in several linkage groups (27), and it is unknown which genes or gene clusters are lacking in NRRL-5918. However, if there is a regulatory gene controlling overall expression of the aflatoxin biosynthetic enzymes, as has

been postulated (7, 21, 29), a lesion in that gene could explain the failure of NRRL-S918 to produce pathway enzymes. Mutations in regulatory loci are potential explanations for atoxigenicity of all the examined strains. Strain AF36 converts norsolorinic acid, the earliest known aflatoxin precursor, to aflatoxin B<sub>1</sub>. This suggests that strain AF36 either is blocked in a structural gene prior to the described portion of the pathway or is mutated at a regulatory locus governing incorporation of acetate units into the aflatoxin polyketide skeleton. The mechanism of atoxigenicity of AF36 clearly differs from that of NRRL-S918.

AF36 and similar strains may prove to be useful tools in the study of aflatoxin biosynthesis because AF36 produces more enzyme activities in the aflatoxin biosynthetic pathway than any of the previously identified atoxigenic strains of either *A. flavus* or *A. parasiticus*. Thus, AF36 may facilitate the identification of new aflatoxin precursors in feeding studies as well as facilitate studies of potential interactions between various aflatoxin precursors. The use of AF36 in such studies may prevent the occurrence of artifacts caused by model systems using unusual media to restrict toxin production in the presence of pathway enzymes (35) and may also preclude the need for radiolabeled precursors in feeding studies with aflatoxin-producing strains of *A. flavus* and *A. parasiticus* (4, 5, 25).

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# U.S. Environmental Protection Agency

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## Aspergillus flavus AF36; Amendment, Temporary Exemption From the Requirement of a Tolerance

OPP-2002-0093; FRL-7183-4 | RIN 2070 Aspergillus flavus AF36; Amendment, Temporary Exemption From the Requirement of a Tolerance AGENCY: Environmental Protection Agency (EPA). ACTION: Final rule.

SUMMARY: This regulation amends an existing temporary exemption from the requirement of a tolerance for residues of the atoxigenic microbial pesticide, Aspergillus flavus AF36 on cotton consistent with the Experimental Use Permit 69224-EUP-1, which will now allow for application to cotton in certain counties in Arizona and Texas.

Interregional Research Project Number 4 (IR-4), on behalf of the USDA/ARS Southern Regional Research Center, submitted a petition to EPA under the Federal Food, Drug, and Cosmetic Act (FFDCA), as amended by the Food Quality Protection Act (FQPA) of 1996, requesting the temporary tolerance exemption amendment. This regulation eliminates the need to establish a maximum permissible level for residues of Aspergillus flavus AF36. The temporary tolerance exemption will expire on December 30, 2004. DATES: This regulation is effective July 17, 2002. Objections and requests for hearings, identified by docket ID number OPP-2002-0093, must be received by EPA on or before September 16, 2002. ADDRESSES: Written objections and hearing requests may be submitted by mail, in person, or by courier. Please follow the detailed instructions for each method as provided in Unit VIII. of the SUPPLEMENTARY INFORMATION. To ensure proper receipt by EPA, your objections and hearing requests must identify docket ID number OPP-2002-0093 in the subject line on the first page of your response. FOR FURTHER INFORMATION

CONTACT: By mail: Shanaz Bacchus, c/o Product Manager (PM) 90, Biopesticides and Pollution Prevention Division (7511C), Environmental Protection Agency, 1200 Pennsylvania Ave., NW., Washington, DC 20460; telephone number: 703-308-6097; e-mail address: [bacchus.shanaz@epa.gov](mailto:bacchus.shanaz@epa.gov). SUPPLEMENTARY INFORMATION: I.

General Information A. Does this Action Apply to Me? You may be affected by this action if you are an agricultural producer, food manufacturer, or pesticide manufacturer. Potentially affected categories and entities may include, but are not limited to:

----- Examples of Categories NAICS codes potentially affected entities -----

Industry 111 Crop production 112 Animal production 311 Food manufacturing 32532 Pesticide manufacturing -----

This listing is not intended to be exhaustive, but rather provides a guide for readers regarding entities likely to be affected by this action. Other types of entities not listed in the table could also be affected. The North American Industrial Classification System (NAICS) codes have been provided to assist you and others in determining whether or not this action might apply to certain entities. If you have questions regarding the applicability of this action to a particular entity, consult the person listed under FOR FURTHER INFORMATION

CONTACT. B. How Can I Get Additional Information, Including Copies of this Document and Other Related Documents? 1. Electronically. You may obtain electronic copies of this document, and certain other related documents that might be available electronically, from the EPA Internet Home Page at <http://www.epa.gov/>. To access this [[Page 46883]] document, on the Home Page select "Laws and Regulations," "Regulations and Proposed Rules," and then look up the entry for this document under the "Federal Register--Environmental Documents." You can also go directly to the Federal Register listings at <http://www.epa.gov/fedrgstr/>. A frequently updated electronic version of 40 CFR part 180 is available at <http://www.access.gpo.gov/nara/cfr/>

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cfhtml 00/Title 40/40cfr180 00.html, ~~ENVIRONMENT~~ a beta site currently under development. 2. In person. The Agency has established an official docket for this action under docket ID number OPP-2002-0093. The official docket consists of the documents specifically referenced in this action, and other information related to this action, including any information claimed as Confidential Business Information (CBI). Interested parties should consult both the documents that are physically located in the docket, as well as the documents that are referenced in those documents. The public version of the official docket does not include any information claimed as CBI. The public version of the official docket, which includes printed, paper versions of any electronic comments submitted during an applicable comment period is available for inspection in the Public Information and Records Integrity Branch (PIRIB), Rm. 119, Crystal Mall #2, 1921 Jefferson Davis Hwy., Arlington, VA, from 8:30 a.m. to 4 p.m., Monday through Friday, excluding legal holidays. The PIRIB telephone number is (703) 305-5805. II. Background and Statutory Authority A. Statutory Authority Section 408(c)(2)(A)(i) of the FFDCA allows EPA to establish an exemption from the requirement for a tolerance (the legal limit for a pesticide chemical residue in or on a food) only if EPA determines that the exemption is "safe." Section 408(c)(2)(A)(ii) defines "safe" to mean that "there is a reasonable certainty that no harm will result from aggregate exposure to the pesticide chemical residue, including all anticipated dietary exposures and all other exposures for which there is reliable information." This includes exposure through drinking water and in residential settings, but does not include occupational exposure. Section 408(b)(2)(C) requires EPA to give special consideration to exposure of infants and children to the pesticide chemical residue in establishing a tolerance and to "ensure that there is a reasonable certainty that no harm will result to infants and children from aggregate exposure to the pesticide chemical residue. . . ." Additionally, section 408(b)(2)(D) requires that the Agency consider "available information concerning the cumulative effects of a particular pesticide's residues" and "other substances that have a common mechanism of toxicity." EPA performs a number of analyses to determine the risks from aggregate exposure to pesticide residues. First, EPA determines the toxicity of pesticides. Second, EPA examines exposure to the pesticide through food, drinking water, and through other exposures that occur as a result of pesticide use in residential settings. B. Factual Background This extension of the temporary exemption from the requirement of a tolerance is associated with an extension of an Experimental Use Permit (69224-EUP-1), which was granted in May 1996 to the Southern Regional Research Center, United States Department of Agriculture, Agricultural Research Service (USDA ARS), 1100 Robert E. Lee Blvd., New Orleans, LA 70179-0687. Both the temporary exemption from tolerance and the Experimental Use Permit in Arizona expire December 30, 2003. In the Federal Register of (March 25 2002, 57 FR 13628) (FRL-6827-8), EPA issued a notice pursuant to section 408 of the FFDCA, 21 U.S.C. 346a, as amended by the FQPA (Public Law 104-170), announcing the filing of an amended pesticide tolerance petition (PP 5E4575) by Interregional Research Project Number 4 (IR-4), New Jersey Agricultural Experiment Station, Technology Center of New Jersey, 681 U.S. Highway #1 South, North Brunswick, NJ 08902-3390 on behalf of the USDA/ARS Southern Regional Research Center, 1100 Robert E. Lee Blvd., P.O. Box 19687, New Orleans, LA 70179. This notice included a summary of the petition prepared by the petitioner, Dr. Michael Braverman. It referred to data previously evaluated and summarized by the Agency as published in the Federal Register of May 26 1999 (64 FR 28371) (FRL-6081-2), and the extension of the temporary tolerance exemption as published in the Federal Register of May 23 2001 (66 FR 28383) (FRL-6781-7). The petition requested that 40 CFR part 180.1206 be amended by establishing a temporary exemption from the requirement of a tolerance for residues of *Aspergillus flavus* AF36 on cotton in certain counties in Texas in addition to the current exemption from temporary tolerance on cotton in Arizona. This petition also, requested that this temporary exemption from a tolerance be extended to December 30, 2005. Several comments were received in favor of the amendment to allow use of the microbial pesticide in Texas. The growers were of the opinion that the use of this active ingredient is likely to reduce the high levels of naturally occurring aflatoxin-producing strain. *Aspergillus flavus* AF36 has been found at a range of less than 1 to approximately 5% in certain regions of Texas. One comment was received requesting the Agency to re-evaluate the science of the proposed program and that the risks associated with the use of the active ingredient be considered before a permanent exemption from a tolerance is issued. The main concerns in this comment were the requirement for uniform standards in the expression of aflatoxin

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levels found in the crop, the practical significance of the proposed treatment method in reducing aflatoxin contamination; and the significance of the host stress in the expression of pathogenicity by *Aspergillus flavus*. Considering each of these points, first, the commenter referred to the mixing of units used to measure aflatoxin contamination. This comment specifically referred to the experimental researcher's reports, which include measurement of aflatoxin levels as micrograms per gram of cottonseed rather than the typical expression of micrograms per kilogram of cottonseed. In data submitted to the Agency, there is no indication that the company was in error or misrepresenting the aflatoxin values. In all cases, EPA is careful to pay close scrutiny to the units of measure in data they review and the implications made from the stated values. Secondly, the efficacy of the pesticidal product to reduce the level of aflatoxin in commercial crops was questioned in the comments. The Agency requires that the company present data to confirm their claim to control a public health hazard. The submitted data are available in the public docket and have been reviewed. These data indicate that when *Aspergillus flavus* AF36 is used, a higher percentage of the treated commodity meets, or is less than, the standards of aflatoxin required by the Food and Drug Administration (FDA), and the aflatoxin contamination in the experimental region is lowered. The growers ultimately decide if the reduced aflatoxin contamination is worth the treatment cost, but all cotton and its by-products sold for food/feed must meet the FDA aflatoxin standard. [[Page 46886]] Regarding testing of the atoxigenic fungus, *Aspergillus flavus* AF36, on stressed or immunosuppressed species to detect any pathogenic potential in plants, insects, or mammals, EPA's guideline requirements are designed to address the normal immune response to microbial exposure. These tests include non-self/foreign recognition and response or clearance by the immune system over time. EPA is examining new methods that may address the potential of a microbe to infect stressed or immunocompromised hosts. In the interim, special measures have been included in the experimental treatments to reduce exposure to *Aspergillus flavus* AF36 outside of the designated treatment areas. The experimental plan also requires extensive data collection to examine the fate and persistence of *Aspergillus flavus* AF36 as a component of the local fungal population. Exposure to *Aspergillus flavus* is inevitable, because the fungus normally occurs in the environment. Given the ubiquitous nature of various strains of *Aspergillus flavus*, the precautions associated with this experimental program, data indicating no undue adverse health effects to test rodent species by oral ingestion of *Aspergillus flavus* AF36, as well as the current FDA monitoring of aflatoxin levels, there is a reasonable certainty of no harm resulting from the use of the non-aflatoxin-producing fungus, *Aspergillus flavus* AF36. III. Toxicological Profile and Risk Assessment Consistent with section 408(b)(2)(D) of the FFDCA, EPA has reviewed the available scientific data and other relevant information in support of this action and considered its validity, completeness, and reliability and the relationship of this information to human risk. EPA has also considered available information concerning the variability of the sensitivities of major identifiable subgroups of consumers, including infants and children. Based on the data and analyses outlined in the Federal Register of May 26 1999 (66 FR 28371), and summarized below, EPA has concluded that there is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to residues of *Aspergillus flavus* AF36 arising from the limited use pattern of the experimental use permit. This includes all anticipated dietary exposures and all other exposures for which there is reliable information. 1. Food. The cultural practice allows application of the microbial pesticide prebloom to cotton. This precludes the potential for direct residues of *Aspergillus flavus* AF36 per se to remain on the treated cotton. Only the seed of the treated commodity, cotton, is likely to be processed as food for cottonseed oil. Residues of *Aspergillus flavus* AF36 or its metabolites are likely to be removed from cotton seed oil during this processing. In addition, the data submitted demonstrate that the proposed strain of *Aspergillus flavus* AF36, has a low toxicity potential, and, therefore, is likely to pose a minimal to non-existent hazard if used as labeled. The acute oral LD<sub>50</sub> of rats treated by gavage for 14 days is greater than 5,000 mg/kg. Further, the proposed strain of *Aspergillus flavus*, AF36, does not produce aflatoxin. Aflatoxin is regulated on the by-products of cotton by the Food and Drug Administration. The May 23 2001 Federal Register Notice also, discusses that no adverse effects were reported in the annual reports of the Experimental Use Permit 69224-EUP-1, and, in some instances, aflatoxin levels of cotton seed were reduced in treated cotton (May 23, 2001, 66 FR 28383). 2. Dermal exposure

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Non-occupational dermal exposure and risk to adults, infants and children are not likely if the pesticide is used as labeled. If the microbe exhibits dermal sensitizing properties which is associated with this genus of fungi, the boundaries are likely to maintain distribution near treated areas thus protecting nearby at-risk populations. To further minimize exposure to immunocompromised or sensitive populations, infants and children, the Agency continues to require that the pesticide must not be applied within a boundary of 400 feet of schools, daycare and health care facilities and hospitals. 3. Inhalation exposure. Based on the method of application to the soil of cultivated cotton fields, prebloom with set boundaries, non-occupational inhalation exposure and risk to human adults, children and infants are likely to be minimal. 4. Determination of safety for U.S. population, infants and children. FFDCA section 408 provides that EPA shall apply an additional tenfold margin of exposure (safety) for infants and children in the case of threshold effects to account for prenatal and postnatal toxicity and the completeness of the data base unless EPA determines that a different margin of exposure (safety) will be safe for infants and children. In this instance, based on the above findings, EPA believes there are reliable data to support the conclusion that there are no threshold effects of concern to infants, children, and adults when *Aspergillus flavus* AF36 is used as labeled, and that no additional margin of exposure is necessary. 5. Cumulative effects. This is the only microbe in the genus *Aspergillus* which is in an experimental use program at this time. *Aspergillus* species are naturally occurring ubiquitous fungi, such that exposure to various species is normal. The data submitted to the Agency support the claim that *Aspergillus flavus* AF36 is non-aflatoxin producing. When applied prior to flowering, *Aspergillus flavus* has been shown to exclude aflatoxin-producing fungi competitively from the developing crop and to reduce aflatoxin contamination of cottonseed. Data show that the proposed use will not result in appreciable increases in the long-term population of *Aspergillus flavus* on the crop beyond naturally occurring levels. Furthermore, there is no expectation of cumulative effects with other pesticides. IV. Other considerations. t. Endocrine disruptors. EPA does not have any information regarding endocrine effects of this microbial pesticide at this time. 2. Analytical methods. Starter cultures are screened on the basis of vegetative incompatibility with the toxigenic strain. *Aspergillus flavus* AP 36 does not demonstrate vegetative compatibility with the aflatoxin-producing S strain. Aflatoxin production is monitored by standard thin layer chromatography (tlc) procedures and visualization via scanning fluorescence densitometry and there is a zero tolerance for aflatoxin. Human pathogens are reported to be within regulatory levels (May 26 1999, 64 FR 28371). Treated cotton and its by-products are screened for aflatoxin prior to introduction into the channels of commerce. FDA does not allow cotton seed products containing aflatoxin above 20 parts per billion (ppb) to be used in dairy rations or above 300 ppb to be used for feeding beef cattle. 3. Codex maximum residue level. There is no codex maximum residue level for *Aspergillus flavus* AF36. V. Objections and Hearing Requests Under section 408(g) of the FFDCA, as amended by the FQPA, any person may file an objection to any aspect of this regulation and may also request a hearing on those objections. The EPA procedural regulations which govern the submission of objections and requests for hearings appear in 40 CFR part 178. Although the procedures in those regulations require some modification to reflect the amendments made to the [[Page 46887]] FFDCA by the FQPA of 1996, EPA will continue to use those procedures, with appropriate adjustments, until the necessary modifications can be made. The new section 408(g) provides essentially the same process for persons to "object" to a regulation for an exemption from the requirement of a tolerance issued by EPA under new section 408(d), as was provided in the old FFDCA sections 408 and 409. However, the period for filing objections is now 60 days, rather than 30 days. A. What Do I Need to Do to File an Objection or Request a Hearing? You must file your objection or request a hearing on this regulation in accordance with the instructions provided in this unit and in 40 CFR part 178. To ensure proper receipt by EPA, you must identify pocket ID number OPP-2002-0093 in the subject line on the first page of your submission. All requests must be in writing, and must be mailed or delivered to the Hearing Clerk on or before September 16, 2002. f. Filing the request. Your objection must specify the specific provisions in the regulation that you object to, and the grounds for the objections (40 CFR 178.25). If a hearing is requested, the objections must include a statement of the factual issues(s) on which a hearing is requested, the requestor's contentions on such issues, and a summary of any evidence relied upon by the objector (40 CFR 178.27). Information submitted in connection with an objection or hearing request may be claimed confidential by marking any part or all of that information as CBI.

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Information so marked will not be disclosed except in accordance with procedures set forth in 40 CFR part 2. A copy of the information that does not contain CBI must be submitted for inclusion in the public record. Information not marked confidential may be disclosed publicly by EPA without prior notice. Mail your written request to: Office of the Hearing Clerk (1900), Environmental Protection Agency, 1200 Pennsylvania Ave., NW., Washington, DC 20460. You may also deliver your request to the Office of the Hearing Clerk in Rm. C400, Waterside Mall, 401 M St., SW., Washington, DC 20460. The Office of the Hearing Clerk is open from 8 a.m. to 4 p.m., Monday through Friday, excluding legal holidays. The telephone number for the Office of the Hearing Clerk is (202) 260-4865. 2. Tolerance fee payment. If you file an objection or request a hearing, you must also pay the fee prescribed by 40 CFR 180.33(i) or request a waiver of that fee pursuant to 40 CFR 180.33(m). You must mail the fee to: EPA Headquarters Accounting Operations Branch, Office of Pesticide Programs, P.O. Box 360277M, Pittsburgh, PA 15251. Please identify the fee submission by labeling it "Tolerance Petition Fees." EPA is authorized to waive any fee requirement "when in the judgement of the Administrator such a waiver or refund is equitable and not contrary to the purpose of this subsection." For additional information regarding the waiver of these fees, you may contact James Tompkins by phone at (703) 305-5697, by e-mail at [tompkins.jim@epa.gov](mailto:tompkins.jim@epa.gov), or by mailing a request for information to Mr. Tompkins at Registration Division (7505C), Office of Pesticide Programs, Environmental Protection Agency, 1200 Pennsylvania Ave., NW., Washington, DC 20460. If you would like to request a waiver of the tolerance objection fees, you must mail your request for such a waiver to: James Hollins, Information Resources and Services Division (7502C), Office of Pesticide Programs, Environmental Protection Agency, 1200 Pennsylvania Ave., NW., Washington, DC 20460. 3. Copies for the docket. In addition to filing an objection or hearing request with the Hearing Clerk as described in Unit VIII.A., you should also send a copy of your request to the PIRIB for its inclusion in the official record that is described in Unit I.B.2. Mail your copies, identified by docket ID number OPP-2002-0093, to: Public Information and Records Integrity Branch, Information Resources and Services Division (7502C), Office of Pesticide Programs, Environmental Protection Agency, 1200 Pennsylvania Ave., NW., Washington, DC 20460. In person or by courier, bring a copy to the location of the PIRIB described in Unit I.B.2. You may also send an electronic copy of your request via e-mail to: [ooo-docket@epa.gov](mailto:ooo-docket@epa.gov). Please use an ASCII file format and avoid the use of special characters and any form of encryption. Copies of electronic objections and hearing requests will also be accepted on disks in WordPerfect 6.1/8.0 or ASCII file format. Do not include any CBI in your electronic copy. You may also submit an electronic copy of your request at many Federal Depository Libraries. B. When Will the Agency Grant a Request for a Hearing? A request for a hearing will be granted if the Administrator determines that the material submitted shows the following: There is a genuine and substantial issue of fact; there is a reasonable possibility that available evidence identified by the requestor would, if established resolve one or more of such issues in favor of the requestor, taking into account uncontested claims or facts to the contrary; and resolution of the factual issues(s) in the manner sought by the requestor would be adequate to justify the action requested (40 CFR 178.32). VI. Regulatory Assessment Requirements This final rule establishes an amended exemption from the temporary tolerance requirement under FFDCA section 408(d) in response to a petition submitted to the Agency. The Office of Management and Budget (OMB) has exempted these types of actions from review under Executive Order 12866, entitled Regulatory Planning and Review (October 4 1993, 58 FR 51735). Because this rule has been exempted from review under Executive Order 12866 due to its lack of significance, this rule is not subject to Executive Order 13211, Actions Concerning Regulations That Significantly Affect Energy Supply, Distribution, or Use (May 22 2001, 66 FR 28355). This final rule does not contain any information collections subject to OMB approval under the Paperwork Reduction Act (PRA), 44 U.S.C. 3501 et seq., or impose any enforceable duty or contain any unfunded mandate as described under Title II of the Unfunded Mandates Reform Act of 1995 (UMRA) (Public Law 104-4). Nor does it require any special considerations under Executive Order 12898, entitled Federal Actions to Address Environmental Justice in Minority Populations and Low-Income Populations (February 18 1994, 59 FR 7629); or OMB review or any Agency action under Executive Order 13045, entitled Protection of Children from Environmental Health Risks and Safety Risks (April 23 1997, 62 FR 19885). This action does not involve any technical standards that would require Agency consideration of voluntary consensus standards pursuant to section 12(d)

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of the National Technology Transfer and Advancement Act of 1995 (NTTAA), Public Law 104-113, section 12(d) (15 U.S.C. 272 note). Since tolerances and exemptions that are established on the basis of a petition under FFDCA section 408(d), such as the amended temporary tolerance exemption in this final rule, do not require the issuance of a proposed rule, the requirements of the Regulatory Flexibility Act (RFA) (5 U.S.C. 601 et seq.) do not apply. In addition, the Agency has determined that this action will not have a substantial direct effect on States, on the relationship between the national government and the States, or on the distribution of power and responsibilities among the various [[Page 46888]] levels of government, as specified in Executive Order 13132, entitled Federalism (August 10 1999, 64 FR 43265). Executive Order 13132 requires EPA to develop an accountable process to ensure "meaningful and timely input by State and local officials in the development of regulatory policies that have federalism implications." "Policies that have federalism implications" is defined in the Executive Order to include regulations that have "substantial direct effects on the States, on the relationship between the national government and the States, or on the distribution of power and responsibilities among the various levels of government." This final rule directly regulates growers, food processors, food handlers and food retailers, not States. This action does not alter the relationships or distribution of power and responsibilities established by Congress in the preemption provisions of FFDCA section 408(n)(4). For these same reasons, the Agency has determined that this rule does not have any "tribal implications" as described in Executive Order 13175, entitled Consultation and Coordination with Indian Tribal Governments (November 6, 2000, 65 FR 67249). Executive Order 13175, requires EPA to develop an accountable process to ensure "meaningful and timely input by tribal officials in the development of regulatory policies that have tribal implications." "Policies that have tribal implications" is defined in the Executive Order to include regulations that have "substantial direct effects on one or more Indian tribes, on the relationship between the Federal Government and the Indian tribes, or on the distribution of power and responsibilities between the Federal Government and Indian tribes." This rule will not have substantial direct effects on tribal governments, on the relationship between the Federal Government and Indian tribes, or on the distribution of power and responsibilities between the Federal government and Indian tribes, as specified in Executive Order 13175. Thus, Executive Order 13175 does not apply to this rule. VII. Submission to Congress and the Comptroller General The Congressional Review Act, 5 U.S.C. 801 et seq., as added by the Small Business Regulatory Enforcement Fairness Act of 1996, generally provides that before a rule may take effect, the agency promulgating the rule must submit a rule report, which includes a copy of the rule, to each House of the Congress and to the Comptroller General of the United States. EPA will submit a report containing this rule and other required information to the U.S. Senate, the U.S. House of Representatives, and the Comptroller General of the United States prior to publication of this final rule in the Federal Register. This final rule is not a "major rule" as defined by 5 U.S.C. 804(2). List of Subjects in 40 CFR Part 180 Environmental protection, Administrative practice and procedure, Agricultural commodities, Pesticides and pests, Reporting and recordkeeping requirements. Dated: June 27, 2002. Janet L. Andersen, Director, Biopesticides and Pollution Prevention Division, Office of Pesticide Programs. Therefore, 40 CFR chapter I is amended as follows: PART 180--[AMENDED] 1. The authority citation for part 180 continues to read as follows: Authority: 21 U.S.C. 321(q), 346(a) and 374. 2. Section 180.1206 is revised to read as follows: Sec. 180.1206 *Aspergillus flavus* AF36. *Aspergillus flavus* AF36 is temporarily exempt from the requirement of a tolerance in or on cotton. The temporary exemption from a tolerance will expire on December 30, 2004, consistent with the Experimental Use Permit 69224-EUP-1 (FR Doc. 02-17869 Filed 7-16-02; 8:45 am) BILLING CODE 6560-50-S

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URL: <http://www.epa.gov/fedrgstr/EPA-PEST/2002/July/Day-17/p17869.htm>

# Effect of Atoxigenic Strains of *Aspergillus flavus* on Aflatoxin Contamination of Developing Cottonseed

P. J. COTTY, Research Plant Pathologist, Southern Regional Research Center, U.S. Department of Agriculture, Agricultural Research Service, New Orleans, Louisiana 70179

## ABSTRACT

Cotty, P. J. 1990. Effect of atoxigenic strains of *Aspergillus flavus* on aflatoxin contamination of developing cottonseed. Plant Dis. 74: 233-235.

Simultaneous inoculation of wounded 28- to 32-day-old cotton bolls with toxigenic and atoxigenic strains of *Aspergillus flavus* led to lower levels of aflatoxin B<sub>1</sub> (B<sub>1</sub>) in the cottonseed at maturity than in bolls inoculated with the toxigenic strain alone. Six of seven atoxigenic strains tested reduced the level of contamination produced by toxigenic strains. Less B<sub>1</sub> was detected when the atoxigenic strain was introduced into the wound 1 day before inoculation with a toxigenic strain than when atoxigenic and toxigenic strains were coinoculated. In contrast, toxin levels at maturity were not reduced when the atoxigenic strain was introduced 1 day after the toxigenic strain. Use of an atoxigenic strain at 10-fold higher spore concentration led to significant reduction in B<sub>1</sub> if the atoxigenic strain was introduced within 16 hr after the toxigenic strain. Atoxigenic strains of *A. flavus* may be useful in biological control of aflatoxin contamination.

Aflatoxins are toxic metabolites of the fungi *Aspergillus flavus* Link:Fr. and *A. parasiticus* Speare [6]. These toxins are potent carcinogens that frequently contaminate agricultural commodities and pose a serious threat to humans and domestic animals [2]. There is great variation among strains of *A. flavus* in the quantity of aflatoxins produced [5,7]; this quantity is independent of a strain's ability to infect and colonize developing cottonseed. Strains of *A. flavus* that do not produce aflatoxins in developing cottonseed can be selected from fungal populations in agricultural fields [5].

Atoxigenic strains of *A. flavus* may have potential as biological control agents for reducing aflatoxin contamination. Several plant diseases have been controlled by applying certain strains of the causal organism. Strains of *Pseudomonas syringae* van Hall or *Erwinia herbicola* (Lohnis) Dye that are without genes for ice nucleation can be used to exclude ice-nucleation active strains and prevent frost injury [10]. The cross-protection phenomenon has been used to control several viral diseases [14]; non-pathogenic strains of *Fusarium oxysporum* Schlechtend.:Fr. can competitively exclude pathogenic strains from infection courts in celery [12]. Similarly, atoxigenic strains of *A. flavus* may be able to exclude toxigenic strains from cotton bolls [5].

The objective of this study was to evaluate atoxigenic strains of *A. flavus*

for their ability to reduce contamination by aflatoxin B<sub>1</sub> (B<sub>1</sub>) in cottonseed maturing in bolls inoculated with toxigenic strains.

## MATERIALS AND METHODS

**Fungal strains and growth conditions.** Strains of *A. flavus* were isolated from agricultural soil and cottonseed collected in Arizona. The origins and aflatoxin-producing capabilities of the strains have been described previously [5]. Strains 13 and 42 produced large quantities of aflatoxins both in culture and in developing cottonseed; strains 19, 36, 40, 51, 53, 55, and 63 did not produce detectable levels (10 ng/g) [5]. Active cultures were grown in the dark at 30°C on a medium containing 5% V-8 juice and 2% agar [5]. For long-term storage, plugs (3 mm in diameter) of sporulating cultures were maintained at 8°C in 4-dram vials containing 5 ml of distilled water [4]. Inoculum was prepared by suspending conidia from 7- to 10-day-old cultures in distilled deionized water.

**Infection of developing cottonseed.** Plants of *Gossypium hirsutum* L. 'Deltapine 90' were grown in a greenhouse in 3-L pots containing a 1:1 mixture of Pro-mix and sand [4]. Plants were fertilized weekly with 100 ml of 2,000 ppm Miracle-Gro beginning 3 wk after emergence. Plants were maintained at all times in complete randomized blocks. At 29-31 days after anthesis, pink bollworm damage was simulated in developing cotton bolls by wounding them once in a single lock using a cork borer (3-mm diameter) to a depth of 3-4 mm [4,8]. Each boll was inoculated by placing a 10- $\mu$ l aliquot of an aqueous suspension of conidia into the wound [4]. Bolls inoculated with two strains received a 10- $\mu$ l aliquot of each strain.

To determine how coinoculation of wounds with toxigenic and atoxigenic strains affects aflatoxin contamination at maturity, each boll was inoculated either with approximately 20,000 conidia of a single strain or with 20,000 conidia of the toxigenic strain followed immediately by 20,000 conidia of the atoxigenic strain. To evaluate how prior colonization of wounds by atoxigenic or toxigenic strains affects the ability of the toxigenic strain to contaminate developing cottonseed, wounds inoculated with one strain were subsequently (after 24 hr) inoculated with 20,000 conidia of a second strain. To test the ability of an atoxigenic strain to influence boll contamination after brief initial infection by a toxigenic strain, bolls were inoculated with 2,000 conidia of a toxigenic strain and then reinoculated in the same wound site after various periods (2, 4, 8, or 16 hr) with 20,000 conidia of an atoxigenic strain.

In all tests, bolls were harvested at maturity (3 wk after inoculation) and dried at 60°C for 2 days. After drying, bolls were kept at room temperature in sealed plastic bags containing silica gel desiccant. Treatments were replicated six to eight times; each replicate consisted of one or two plants (one to three bolls). Experiments were performed twice.

**Aflatoxin analyses.** The B<sub>1</sub> content of intact inoculated locules was determined by a modification of the method of the Association of Official Analytical Chemists [13] as previously described [5]. Intact locks were hammered to pulverize the seed and added to 200 ml of acetone and water (85:15). The mixture was shaken for 15 sec, allowed to set overnight, and then filtered through Whatman No. 4 filter paper. A 100-ml portion of the filtrate was mixed with 100 ml of an aqueous solution of 0.22 M Zn (CH<sub>3</sub>COO)<sub>2</sub> and 0.008 M AlCl<sub>3</sub>. Diatomaceous earth (5 g) was added to the mixture, which was shaken and left to settle for 1-2 hr. The liquid phase was filtered (Whatman No. 4 filter paper) and 100 ml of the filtrate was extracted twice with 25 ml of methylene chloride. The hydrophobic fractions were pooled and dried; residues were dissolved in methylene chloride. B<sub>1</sub> was purified by thin-layer chromatography and quantified with a densitometer with fluorescence capabilities [13].

Statistical analysis. Analyses were performed either manually or with the

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Statistical Analysis System (SAS Institute, Inc., Cary, NC). All multiple comparisons were first subjected to analysis of variance. Toxin values were log-transformed ( $\log x + 1$ ) when necessary to homogenize variances among treatments.

Treatment replicates from two experiments were ranked, and the ranks were subjected to split-plot analyses as follows. In tests comparing atoxigenic strains, the test was the main plot and the strain was the subplot. In tests evaluating the effect of challenge with an atoxigenic strain after brief infection by a toxigenic strain, the test was the main plot and the treatment (no challenge or challenge after 2, 4, 8, or 16 hr) was the subplot. Significant differences among treatment means were determined with the LSD test for split-plot analyses (11).

## RESULTS

Very high concentrations of B1 were detected in seed from bolls inoculated with strain 13 or strain 42 (Table 1). However, strain 13 produced significantly more toxin than strain 42. In contrast, bolls coinoculated with conidia of toxigenic and atoxigenic strains in equal proportions had markedly reduced quantities of B1 in their seed at maturity. The magnitude of the reduction in toxin associated with coinoculation with strain 36 appeared proportionally greater with strain 42 than with strain 13 (Table 1). The occurrence of aflatoxin was prevented almost completely by introducing strain 36 into wounds 1 day before inoculation with an equal quantity of conidia of strain 13 or strain 42 (Table 1). Seeds from bolls inoculated with a toxigenic strain 1 day before inoculation with strain 36 contained B1 levels equal to that of seed from bolls inoculated with the toxigenic strain alone.

In both the test of different atoxigenic strains and the test of delayed challenge, the test variable was not significant ( $P = 0.05$ ), and it did not interact with the treatment variable (Tables 2 and 3). Consequently, data from the two tests were pooled for each experiment. In both cases, the treatment variable was significant ( $P = 0.05$ ).

Six of seven atoxigenic strains significantly reduced accumulation of

aflatoxin in bolls inoculated with the highly toxigenic strain 13 (Table 2). Strain 36 was the most effective at limiting contamination by strain 13. Inoculation of developing cotton bolls with strain 36 alone usually resulted in aflatoxin-free cottonseed at maturity. However, low levels (<50 ng/g) of B1 were occasionally extracted from such seed (data not shown).

When bolls were inoculated with toxigenic strain 13 and then reinoculated (in the same wound site after various time periods) with 10-fold more conidia of atoxigenic strain 36, they developed lower aflatoxin levels than bolls inoculated with strain 13 alone (Table 3). The quantity of B1 in cottonseed at maturity was significantly ( $P = 0.05$ ) reduced when strain 36 was inoculated into bolls up to 16 hr after inoculation with strain 13 (Table 3).

## DISCUSSION

Atoxigenic strains of *A. flavus* appear to have potential as biological control agents for reducing aflatoxin contamination in cottonseed. Atoxigenic strains are endemic to agricultural fields and should be equally adapted to the hot, dry conditions that favor host colonization and infection by toxigenic strains. Typically, *A. flavus* comes in contact with crops before harvest and remains associated with the crop throughout harvest and storage (9). Thus, seed can become contaminated with B1 both before and after harvest (9). Atoxigenic strains should be able to proliferate under the same conditions as toxigenic strains and, once applied in sufficient quantity, they should have activity proportional to need throughout the season and during storage. These characteristics indicate a potential use for atoxigenic strains of *A. flavus* in a biocontrol strategy for managing aflatoxin contamination.

Populations of *A. flavus* in agricultural fields are composed of strains that vary widely in aflatoxin-producing ability, sclerotial size, and virulence (5). Atoxigenic strains of *A. flavus* also appear to vary in their ability to prevent aflatoxin contamination of cottonseed. Screening of field populations of *A. flavus* may result in strains more efficient at preventing aflatoxin contamination.

The occasional occurrence of low levels of aflatoxins in bolls inoculated only with atoxigenic strain 36 may have been caused by chance introduction of a toxigenic strain into the wounded boll before or during inoculation. Such introduction is likely because *A. flavus* sporulates profusely on inoculated bolls and several experiments were performed simultaneously in the same greenhouse. However, we cannot rule out the possibility that some atoxigenic strains are unstable or that some strains can produce toxin under certain conditions. In a given crop, relatively few seeds contain large concentrations of toxin; these seeds typically account for the majority of toxin within a sample (1). Therefore, occasional low levels (<50 ng/g) of toxin produced by biocontrol strains should not prevent them from reducing these high aflatoxin levels and being useful in the management of aflatoxin contamination. Strain stability, however, should be an important criterion in selection of bio-

Table 2. Effect of various atoxigenic strains of *Aspergillus flavus* on aflatoxin contamination of cottonseed by a toxigenic strain<sup>1</sup>

Atoxigenic strain	Aflatoxin B <sub>1</sub> (μg/g) <sup>2</sup>
None	66.24 a
53	35.47 ab
51	20.32 b
19	12.52 bc
55	6.71 bc
63	5.86 bc
40	3.31 bc
36	0.65 c

<sup>1</sup> Developing cotton bolls were inoculated first with toxigenic strain 13 and 30 min later with an atoxigenic strain.

<sup>2</sup> Values are averages of eight observations made during two tests. Values followed by the same letter are not significantly different by the LSD test for split-plot analyses (11). Analyses were performed on ranks assigned to values within tests before analysis.

Table 3. Effect of challenge with an atoxigenic strain on production of aflatoxin in developing cottonseed by a toxigenic strain of *Aspergillus flavus*<sup>1</sup>

Time between inoculation and challenge (hr)	Aflatoxin B <sub>1</sub> (μg/g) <sup>2</sup>
2	1.40
4	1.51
8	3.69
16	6.89
No challenge	30.35

<sup>1</sup> Developing cotton bolls were inoculated first with toxigenic strain 13 and then after various periods with a 10-fold greater quantity of conidia of atoxigenic strain 36.

<sup>2</sup> Values are averages of eight observations made during two tests. Values for 2, 4, 8, and 16 hr differ significantly ( $P = 0.05$ ) from no challenge but not from each other. Analyses were performed on ranks assigned to values within tests before analysis.

Table 1. Aflatoxin content of cotton bolls inoculated with toxigenic and atoxigenic *Aspergillus flavus* strains individually and in combination

Strain	Toxicity	Aflatoxin B <sub>1</sub> content of cottonseed (μg/g) <sup>1</sup>			
		Inoculated alone	Coinoculated with strain 36	Inoculated 24 hr after strain 36	Inoculated 24 hr before strain 36
13	+	72 w	6 x	0.4 z	96 w
42	+	17 y	0 z	0.0 z	15 y
36	-	0 t	...	...	...

<sup>1</sup> Limit of detection: 10 ng/g. Values are means of eight replicates. Means followed by the same letter do not differ significantly ( $P = 0.05$ ) by Fisher's least significant difference test. Data was log-transformed before analysis.

control strains. Efforts to produce genetically altered strains without potential to produce aflatoxins should be encouraged.

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## Reduction in Aflatoxin Content of Maize by Atoxigenic Strains of *Aspergillus flavus*

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### ABSTRACT

In field plot experiments, an atoxigenic strain of *Aspergillus flavus* interfered with preharvest aflatoxin contamination of corn when applied either simultaneously with or one day prior to a toxigenic strain. The atoxigenic strain reduced preharvest aflatoxin contamination 80 to 95%. The atoxigenic strain was also effective in reducing postharvest aflatoxin contamination caused by both an introduced toxigenic strain and by strains resident on the kernels. The results suggest that atoxigenic strains of *A. flavus* may have potential use as biological control agents directed at reducing both preharvest and postharvest aflatoxin contamination of corn.

Aflatoxins, toxic metabolites of the fungi *Aspergillus flavus* Link: Fr. and *A. parasiticus* Speare, are potent carcinogens which pose serious health hazards to humans and domestic animals because they frequently contaminate agricultural commodities (4,8). Corn grown in the southeastern United States is more frequently colonized by high populations of *A. flavus* than corn grown in the Midwest, where most U.S. corn is grown (19). However, the drought of 1988 created favorable conditions for *A. flavus* in the Midwest, and the Wall Street Journal (February 23, 1989) reported one-third of the crop tested in Iowa and Illinois contained dangerous levels of aflatoxin. This, according to the article, caused greatly increased concern in the U.S. corn industry.

Biological control of several plant diseases has been demonstrated by utilizing certain strains of the causal organism (12,14,15,20). Strains of *A. flavus* that do not produce aflatoxins have been selected from fungal populations in cotton and corn fields (6,17). In greenhouse tests, atoxigenic strains of *A. flavus* significantly reduced production of aflatoxin B<sub>1</sub> in cottonseed coinoculated during development with toxigenic strains (7). When atoxigenic strains were introduced into wounded cotton bolls one day prior to the toxigenic strain, even greater control of aflatoxin was obtained (7). This study provided useful information but was carried out in a controlled environment. In the field, where *A. flavus* first associates with the crop, greater biological and environmental complexities exist.

Also, it is not known if atoxigenic strains influence contamination of harvested crops which already possess complex microflora and may be previously infected with *A. flavus* (10). In the present study, an atoxigenic strain, previously identified and shown effective in greenhouse tests on cottonseed, was tested for efficacy on corn under field and storage conditions. A preliminary presentation of these studies has been made (3).

### MATERIALS AND METHODS

#### Fungal strains and growth conditions.

Strains of *A. flavus* utilized in this study were isolated from agricultural soil and cottonseed in Arizona (6,7). Strain 13 produced large quantities of aflatoxins both in culture and in developing cottonseed, while strain 36 did not produce detectable levels (<10 ng/g) (6). Cultures were grown at 30°C in the dark on a 5% V-8 juice, 2% agar medium. Plugs (3 mm in diameter) of sporulating cultures were stored at 8°C on a long-term basis in 4-dram vials containing 5 ml of distilled water (5,6). Conidia from 7- to 10-d-old cultures suspended in deionized water served as inocula (7).

#### Inoculation of developing corn kernels

Field corn (Pioneer Brand 3369A) planted on 76.2-cm centers in a silty-loam type soil in New Orleans, LA was utilized in these experiments. Ten d after the 50% silk stage, each developing ear was wounded once with a cork borer (3 mm diameter) to a depth of 5 mm. Each ear was inoculated by applying 20 µl of a spore suspension (4.0 × 10<sup>8</sup> conidia/ml) to a wound.

The treatments were applications of either the toxigenic or atoxigenic strains alone, the toxigenic strain followed immediately by the atoxigenic strain or the atoxigenic strain 24 h after the toxigenic strain. Wounded, uninoculated ears were used as additional controls. Treatments were replicated six times (3 ears/replicate) and organized into randomized complete blocks. Experiments were performed twice (tests 1 and 2). Corn used in the first field experiment was planted in mid-April and harvested in mid-July (1989); corn for the second experiment was planted in early May and harvested in early August (1989).

In all experiments, ears were harvested at maturity and dried in a forced-air oven at 60°C for 2 d. After drying, ears were kept at room temperature in sealed plastic bags containing silica gel desiccant until aflatoxin analysis.

#### Inoculation of harvested corn kernels

Ears harvested at maturity from uninoculated portions of the above test plots were shelled and the kernels dried at 60°C for 2 d in a forced-air oven. After drying, kernels were kept at room temperature in sealed plastic bags containing silica gel desiccant until used. The initial moisture content of the kernels was determined according to the official method of the American Oil Chemists Society (2).

Ten g of kernels in 50-ml Erlenmeyer flasks were either inoculated with a single fungal strain, or a mixture (1:1) of the toxigenic and atoxigenic strains or hydrated with sterile, deionized water alone. Conidia for each inoculation, approximately  $4.0 \times 10^6$  per strain tested, were suspended in the amount of deionized water needed to bring kernel moisture content to 22% upon application. Flasks were subsequently sealed with Styrofoam plugs, covered with aluminum foil, and incubated at 28°C for 12 d. The contents were then dried in a forced-air oven at 60°C for 2 d to halt fungal activity and prepare the sample for aflatoxin analyses. In a second set of postharvest experiments, the above protocol was altered to simulate aspects of postharvest storage. In these tests, all inoculum suspensions and water controls contained a surfactant (0.02% Tween 80) to improve seed coverage. After either inoculation with a single fungal strain or hydration to 22% moisture content with deionized water, kernels were incubated for 24 h at 28°C and then dried at 45°C for 3 d in a forced-air oven. Dried kernels were stored 8 d at 28°C and then rehydrated to 22% with either a suspension of spores ( $4.0 \times 10^6$ ) of a toxigenic strain or distilled water. After rehydration, flasks were incubated for 12 d at 28°C, dried, and analyzed for aflatoxins.

All experiments were performed twice and replicated six times; each replicate consisted of one flask.

#### Aflatoxin analyses

The aflatoxin B<sub>1</sub> content of replicates from both the preharvest and the postharvest studies was determined with official methods of the American Oil Chemists Society (1). Toxin was identified by thin layer chromatography (TLC) and quantified directly on the TLC plates with a scanning densitometer with a fluorometry attachment (Model CS-930; Shimadzu Scientific Instruments, Inc., Tokyo, Japan). In the case of the preharvest tests, each replicate/sample to be analyzed contained 45 kernels (12 to 13 g), 15 from

each of three ears. The 15 kernels were chosen by first identifying the kernel which lined up with the wound in the husks. This "central" kernel was then used to identify the 14 adjacent kernels which consisted of one column in the left and right and two rows to the top and bottom.

#### Statistical analysis

Analyses were performed with the Statistical Analysis System (SAS Institute Inc., Cary, NC). Treatment replicates from each experiment were ranked, and ranks were subjected to split-plot analyses where the test was the main plot and the treatment was the subplot. Differences among treatment means were determined by the least significant difference test. All multiple comparisons were first subjected to analysis of variance.

## RESULTS

In preharvest experiments, the test variable was not significant ( $P=0.05$ ) and did not interact with the treatment variable. This allowed data from both tests to be pooled (Table 1). Very high levels of aflatoxin B<sub>1</sub> were detected in kernels from ears inoculated with the toxigenic strain in the field. Coinoculation with the atoxigenic strain significantly reduced B<sub>1</sub> quantities, as did inoculation with the atoxigenic strain 24 h in advance.

Aflatoxin B<sub>1</sub> levels in kernels coinoculated with the toxigenic and atoxigenic strains after harvest were also significantly lower than levels detected in kernels inoculated with the toxigenic strain alone (Table 1). Also, as in the preharvest experiments, the test variable was not significant ( $P=0.05$ ) and did not interact with the treatment variable, allowing data from both tests to be pooled. Significantly less B<sub>1</sub> was produced postharvest in kernels inoculated with the atoxigenic strain alone than in the uninoculated control.

In postharvest tests where harvested kernels were inoculated with the atoxigenic strain before the toxigenic strain (Table 1), a significant ( $P=0.05$ ) interaction between

TABLE 1. Aflatoxin content of corn kernels wound-inoculated in the field or inoculated after harvest with atoxigenic and toxigenic strains of *A. flavus*.

Treatment	Test 1	Preharvest		Aflatoxin B <sub>1</sub> content of kernels (µg/g) <sup>a</sup>			Postharvest prior inoculation <sup>b</sup>	
		Test 2	Combined	Test 1	Test 2	Combined	Test 1	Test 2
Toxigenic	3,045	2,146	2,595 a	16,044	13,534	14,789 a	30,385 a	23,976 a
Atoxigenic	9	0	5 c	14	186	100 d	218 c	14,040 b
Control-uninoculated	0	0	0 c	1,046	1,254	1,150 c	135 c	6,715 b
Atoxigenic <sup>c</sup> before toxigenic	101	179	140 b	NA <sup>d</sup>	NA	NA	5,118 b	7,844 b
Coinoculation	184	912	548 b	3,909	2,628	3,269 b	NA	NA

<sup>a</sup>Values followed by the same letter are not significantly different by the least significant difference test.

<sup>b</sup>Values for replicates from tests 1 and 2 were not combined because a significant interaction occurred between the test and treatment variables.

<sup>c</sup>Kernels were inoculated with the atoxigenic strain 24 h before the toxigenic strain in preharvest prior inoculation experiments. In postharvest prior inoculation experiments, kernels were inoculated with the atoxigenic strain, incubated for 24 h, then dried and stored for 8 d, before inoculation with the toxigenic strain.

<sup>d</sup>NA = Not applicable.

the test and treatment variables occurred. Thus, data from these two tests were not combined. Inoculation with the atoxigenic strain prior to the toxigenic strain significantly reduced  $B_1$  in both tests when compared to kernels inoculated with the toxigenic strain alone. In test 1, levels of  $B_1$  in the atoxigenic and uninoculated control treatments were significantly lower than in the kernels inoculated with the toxigenic strain after the atoxigenic strain. However, in test 2, the levels detected in these three treatments did not differ significantly.

## DISCUSSION

Populations of *A. flavus* in agricultural fields are composed of strains that vary widely in aflatoxin-producing ability, and this ability is apparently unrelated to a strain's potential to infect and colonize host tissues (6,7). These observations suggest that naturally occurring atoxigenic strains may be able to outcompete toxigenic strains during infection of developing crops and thereby prevent aflatoxin contamination (6). Several fungi can interfere with aflatoxin production on artificial media or other sterile substrates (9,13,16). However, the biocontrol potential of those fungi tested in developing corn, grown under controlled conditions, is inadequate in preventing aflatoxin production by *A. flavus* (18). Greenhouse experiments verified the biocontrol potential of atoxigenic *A. flavus* strains on cotton (7). Part of the attraction of controlling aflatoxin contamination with strains of *A. flavus* relates to the theoretical ability of the atoxigenic strains to be active under the same environmental conditions where *A. flavus* toxigenic strains are active (7). This ability was tested in the field experiments presented here and the results suggest this speculation may have merit. The atoxigenic strain of *A. flavus* was very effective at preventing contamination of corn under field conditions favoring high levels of aflatoxin contamination.

The atoxigenic strain used in these studies was isolated from soil collected in a cotton field in Arizona. There may be some adaptation of *A. flavus* strains to particular hosts or regions. If adaptation does exist, even greater control could be provided by atoxigenic strains obtained through the screening of corn field populations of *A. flavus* from particular growing regions.

*A. flavus* comes in contact with corn kernels prior to harvest and remains with the crop throughout harvest, storage, and even use (11). The potential for aflatoxin contamination thus exists both before and after harvest (11). The atoxigenic strain reduced contamination by 76 to 81% when shelled kernels were coinoculated with toxigenic and atoxigenic strains. Strain efficacy was also demonstrated in the experiment where dried kernels were exposed to a simulated breakdown in storage conditions. These observations indicate potential use of atoxigenic *A. flavus* strains to prevent postharvest infection and subsequent contamination by toxigenic strains. In two out of four postharvest tests, there was a significant decrease in aflatoxin detected in kernels inoculated with the atoxigenic strain, when compared with the uninoculated control. This may indicate postharvest application of atoxigenic strains

has potential to prevent postharvest contamination of corn by strains associated with the crop in the field. Failure to see reductions in all four tests may be due to interference from uncontrolled microbial activity. Since experimental conditions were designed to exclude sterilization, kernel microflora may have differed considerably in each test.

Very low amounts of aflatoxins were detected in corn inoculated in the field with the atoxigenic strain alone. Toxin was present in only one replicate in one test. This contamination may be due to chance introduction of a toxigenic strain endemic to the field.

In test 2 of the postharvest prior inoculation experiment, atoxigenic strain efficacy was significant but not as great as in other tests (Table 1). This apparent reduced efficacy is due to a single high value.

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**STABILITY OF MODIFIED *ASPERGILLUS FLAVUS*  
COMMUNITIES: NEED FOR AREA-WIDE  
MANAGEMENT**

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Abstract

*Aspergillus flavus*, the causal agent of aflatoxin contamination of cottonseed, is a complex species composed of many distinct vegetative compatibility groups. Isolates belonging to different vegetative compatibility groups may produce widely different quantities of aflatoxins. Some naturally occurring isolates of *A. flavus* produce no aflatoxins. Some of these atoxigenic strains have the ability to competitively exclude aflatoxin-producing strains during crop infection and thereby reduce aflatoxin contamination. In both greenhouse and field-plot tests atoxigenic strain efficacy has repeatedly been demonstrated. *A. flavus* communities resident in soils vary among agricultural fields in aflatoxin producing capacity. Field-plot tests suggested that applications of atoxigenic strains may provide long-term reductions in the aflatoxin producing potential of fungi resident in treated fields. Tests to evaluate the longevity of changes to *A. flavus* communities induced by atoxigenic strain applications were initiated in 1996 under an Experimental Use Permit (EUP) issued by the EPA. The experimental program outlined in the EUP called for treatments over a three year period (1120 acres total) and for monitoring the *A. flavus* community from 1996 through 1999. Different treatment regimes were applied to different fields with some fields receiving treatment only in a single year and others receiving treatments in multiple years.

Sterile wheat seed colonized by an atoxigenic strain was applied to 22 fields ranging in size from 10 to 160 acres from 1996 to 1998. The material was applied either by air or ground at the rate of 10 lb. per acre. Crops were treated only once. In order to monitor changes to the composition of *A. flavus* communities, soil samples were collected prior to application each year. From 1996 through 1999 over 8,000 isolates of *A. flavus* were cultivated from soil samples taken from the treatment areas. Isolates were characterized by strain and those assigned to the L strain of *A. flavus* were further characterized by vegetative compatibility analysis in order to determine applied strain distribution.

One year after application, atoxigenic strain incidence was greatly increased and incidence of the highly toxigenic S strain was greatly decreased in all treated and many adjacent

fields. The applied strain incidence gradually declined by the second year after application. However, even with this decline, the atoxigenic strain remained in treated fields at levels significantly higher than prior to treatment. The incidence of the applied strain in fields adjacent to treated fields was variable indicating possible directional movement of the strain from treated to untreated fields. In some locations crop and crop stage were apparently important determinants of influences beyond treated fields.

One of three fields treated in 1996 was not subsequently treated. Incidence of the atoxigenic strain went from 1.8% prior to treatment to 96% one year after, 52% two years after, and 47% three years after treatment. Long-term influences on the incidence of the S strain also occurred with a 52% incidence pretreatment and only a 2% incidence three years after application. Overall, the results suggest that long-term useful reductions in the aflatoxin-producing potential of fungal communities can be achieved by atoxigenic strain application.

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# Influence of Field Application of an Atoxigenic Strain of *Aspergillus flavus* on the Populations of *A. flavus* Infecting Cotton Bolls and on the Aflatoxin Content of Cottonseed

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## ABSTRACT

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An atoxigenic strain of *Aspergillus flavus* was applied to soils planted with cotton in Yuma, Arizona, to assess the ability of the atoxigenic strain to competitively exclude aflatoxin-producing strains during cotton boll infection and thereby prevent aflatoxin contamination of cottonseed. In both 1989 and 1990, the atoxigenic strain displaced other infecting strains during cotton boll development. Displacement was associated with significant reductions in the quantity of aflatoxins contaminating the crop at maturity. Although frequency of infected locules differed between years (1% versus 25%), in both years displacement occurred without increases in the amount of infection as measured by the quantity of locules with bright-green-yellow-fluorescence (BGYF). In the low infection year (1990), locules exhibiting BGYF were analyzed individually for both incidence of the applied strain and aflatoxin content. In the high infection year

(1989), infected seed from each replicate plot (32 total) were pooled and analyzed for both aflatoxin and incidence of the released strain. Results of the latter analyses indicate an inverse relationship ( $r = 0.71$ ,  $P < 0.001$ ) between aflatoxin content and the percent seed infected by the applied strain. In 1990, quantities of *A. flavus* on mature crop surfaces did not differ between treated and untreated plots. When reisolated from the infected crop the applied atoxigenic strain retained the atoxigenic phenotype. Most infecting strains belonging to other vegetative compatibility groups did produce detectable quantities of aflatoxin  $B_1$  in liquid fermentation. The applied atoxigenic strain spread from treated plots to untreated controls at different rates in the two years and accounted for 7 and 25% of *A. flavus* strains isolated from infected locules in untreated control plots in 1990 and 1989, respectively. The results suggest that the aflatoxin-producing potential of *A. flavus* populations associated with crop production can be reduced in order to reduce aflatoxin contamination.

*Additional keywords:* biocompetition, biological control, population displacement.

Aflatoxins are toxic, carcinogenic fungal metabolites produced by certain isolates of the species *Aspergillus flavus* Link:Fr., *A. parasiticus* and *A. nomius* (37). Concern for human and animal health has led to regulatory limitations on the quantity of aflatoxins permitted in foods and feeds throughout most of the world (40). The most toxic and highly regulated aflatoxin is  $B_1$  (40,33). Aflatoxin contamination has long been a concern for several U.S. crops and for animal industries that depend on susceptible crops for feed (33). Whole cottonseed and cottonseed products are commonly fed to various livestock, including dairy cows. Aflatoxins in contaminated seed can be readily transferred to milk in slightly modified form (32,35). U.S. regulations prohibit aflatoxin concentrations over 0.5  $\mu\text{g}/\text{kg}$  in milk. Dairies producing milk tainted with unacceptable aflatoxin levels can have milk destroyed and entire operations temporarily shut down and quarantined (26). To prevent unacceptable aflatoxin levels in milk, the regulatory threshold for aflatoxin  $B_1$  in cottonseed fed to dairy cows is 20  $\mu\text{g}/\text{kg}$  (32,33).

Populations of the primary causal agent of aflatoxin contamination of cottonseed, *A. flavus*, are highly complex and composed of strains that differ morphologically, physiologically, and genetically (4,6,14). Differences among strains in ability to produce aflatoxins are well known (24) and aflatoxin-producing ability is not correlated with strain ability to colonize and infect cotton, *Gossypium hirsutum* L. (14). These observations led to the sugges-

tion that atoxigenic strains of *A. flavus* might be used to exclude toxigenic strains through competition during infection of developing crops and thereby prevent aflatoxin contamination (14,20). In both greenhouse and field experiments, wound inoculation of developing cotton bolls and corn ears with toxigenic and atoxigenic strains simultaneously led to reductions in aflatoxin contamination of the developing crop parts as compared with controls inoculated with only the toxigenic strains (9,15). Atoxigenic strains were effective at preventing postharvest aflatoxin contamination both when the crop was infected naturally in the field and when inoculated after harvest (9). Similarly, in special environmental control plots, peanuts were protected from preharvest aflatoxin contamination by irrigating the developing crop with conidial suspensions of *A. parasiticus* strains that accumulate specific aflatoxin precursors (i.e. O-methylsterigmatocystin and versicolorin-A) but not aflatoxins (25).

Aflatoxin contamination of cottonseed can be minimized by early harvest, prevention of insect damage, and proper storage (17,18). However, even under careful management, unacceptable aflatoxin levels may occur from unpreventable insect damage to the developing crop (22) or from exposure of the mature crop to moisture either prior to harvest (18), or during storage in modules (36), handling, transportation, or even use (17). Competitive exclusion of aflatoxin-producing strains of *A. flavus* with atoxigenic strains of the same fungal species may provide a single method for preventing aflatoxin accumulation throughout crop production and utilization (11,14,15,20).

In the United States, aflatoxin contamination of cottonseed is most consistent and severe in the irrigated western desert valleys where most contamination is associated with pink bollworm dam-

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age (17,22). Contamination levels are highly variable within fields, plants, and even bolls (17,22,30) and contamination is often associated with bolls exhibiting bright-green-yellow-fluorescence (BGYF) on the lint under ultraviolet light (2). BGYF occurs when kojic acid produced by *A. flavus* reacts with peroxidases in developing cotton bolls (31); therefore, BGYF indicates boll infection by *A. flavus* prior to boll maturity via wounds (i.e., pink bollworm exit holes) or infection of partially open bolls (18,28). Because bolls infected through wounds during development accumulate very high aflatoxin levels (13,22), when BGYF is detected, most aflatoxin contamination is associated with the component of the crop exhibiting BGYF (2,18,36). During seasons when aflatoxin contamination is severe, *A. flavus* populations increase as the cotton crop is produced (29). In theory, application of an atoxigenic *A. flavus* strain early in the season should permit the atoxigenic strain to compete with resident toxigenic strains both during crop infection and during population increases associated with cultivation (11). Results of greenhouse studies suggest that the end result of this competition might be reduced aflatoxin in the crop (9,15,21). The current study sought to determine efficacy of an atoxigenic strain in preventing aflatoxin contamination of cottonseed produced in an irrigated desert valley in western Arizona. Summaries of preliminary aspects of this work have been published (16,19).

## MATERIALS AND METHODS

**Cultures and inoculum preparation.** Atoxigenic *A. flavus* strain AF36, previously shown in greenhouse tests to exclude aflatoxin-producing strains competitively during infection of developing cotton bolls was used in all field tests (15,21). Active cultures were maintained in the dark at 32 C on a modified V8 vegetable juice medium (5:2 agar, 5% V-8 vegetable juice, 2% agar, pH

5.2) (14). For long-term storage, plugs (3 mm in diameter) of sporulating cultures were submerged in 5 ml of sterile distilled water and kept at 4 C (14).

Two types of inoculum were produced. Conidial suspensions were produced from 14-day-old cultures grown as above. Plates were flooded with 0.01% w/v Triton X-100, the colony surface was agitated with a rubber policeman to dislodge the spores, and spore concentrations were determined with a hemacytometer. Suspensions were diluted to  $2 \times 10^7$  spores/ml in 0.01% Triton X-100. The second form of inoculum was autoclaved wheat seed that had been colonized by AF36 (20). Whole red winter wheat was purchased from a health food store, autoclaved (1 h, 120 C), allowed to set at room temperature for 18 h, and autoclaved again. Wheat was dried in culture bottles with loose caps in a forced air oven at 60 C for 2 days. The wheat was then seeded with AF36 (approximately 200,000 spores per milliliter) in sufficient water to bring the moisture level of the wheat to between 20 and 25% (w/w). Subsequently, 220 g of wheat was incubated in each 490-cm<sup>2</sup> roller bottle (Corning, Inc., Corning, NY) on a roller drum (5 RPM, 28 C, 7 days). During this incubation the fungus grew in the folds of the seed and under the seed coat but very few or no spores were produced and the appearance of the wheat remained unchanged.

**Field plots.** In both 1989 and 1990, at the Yuma Valley Agricultural Center near Yuma, AZ, cotton (cv. Deltapine 90) was planted in mid-March (9 March 1989 and 14 March 1990) on a silty clay loam soil in rows on 1-m centers. In both years, fields were furrow-irrigated eight times including a preplant irrigation. The experimental design in 1989 was a randomized complete block design augmented with an additional untreated control and

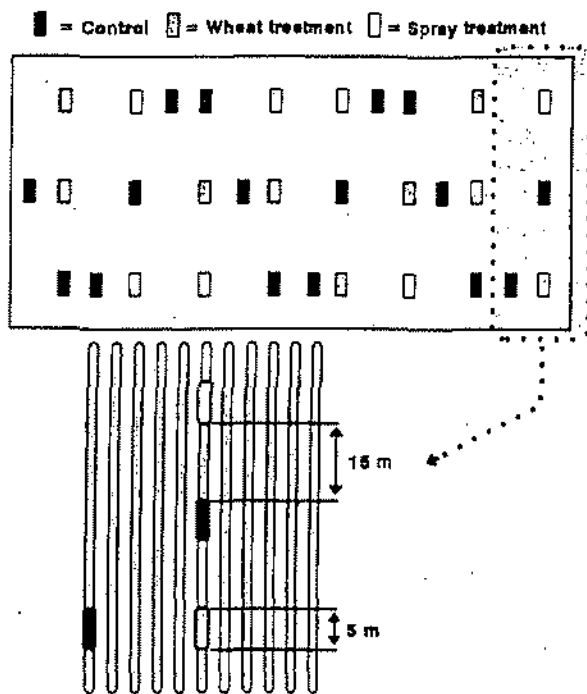


Fig. 1. Field plot design of the 1989 experiment, which contained two untreated controls and eight replicate blocks. Each replicate block was 76 m long and 11 rows wide (1 m centers). Wheat = area where autoclaved wheat seed colonized by *A. flavus* strain AF36 was applied; Spray = area where a conidial suspension of AF36 was applied; Control = sampling area for untreated control.

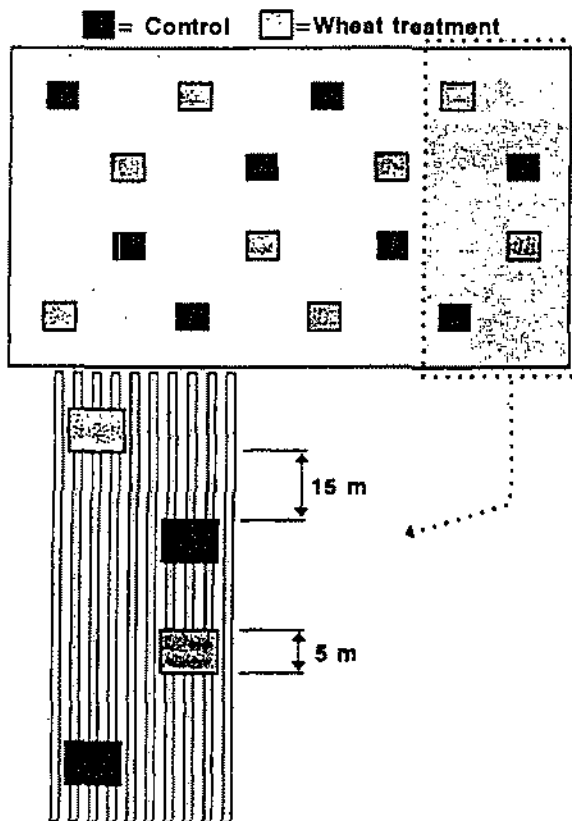


Fig. 2. Field plot design of the 1990 experiment, which contained only one treatment and one untreated control. Each replicate block was 75 m long and 8 rows wide (1 m centers). Wheat = area where autoclaved wheat seed colonized by *A. flavus* strain AF36 was applied; Control = sampling area for untreated control.

replicated eight times (Fig. 1). Each block contained 11 76-m rows of cotton and only the center row was treated. Treatments were applied to 5 m of row and were separated within the row by 15 m of untreated cotton. A second untreated control, designated control 2, was positioned in the first row of each block (Fig. 1). In 1990 the blocks were reduced to three rows and only the wheat treatment was used (Fig. 2). The treated areas were 5 m long and three rows wide and only the center row of each treatment was sampled. The blocks were separated by two untreated rows. Treatments in blocks 1, 3, 5, and 7 started 5 m into the field and were separated by 55 m of untreated cotton; treatments in replicate blocks 2, 4, 6, and 8 started 25 m into the field and were separated by 15 m of untreated cotton (Fig. 2).

Fields used in the 2 yr were 1.2 km apart. The field used for the 1989 test had been planted with cotton for 2 yr immediately prior to the test and a winter fallow was maintained. In both prior years greater than 15% of the bolls were infested with pink bollworms and harvest was delayed, permitting pink bollworm diapause. The average aflatoxin B<sub>1</sub> content of the cottonseed crop produced in this field exceeded 1,000 µg/kg in both prior years. The field used for the 1990 test was planted to winter vegetables immediately prior to the test. Practices typical of commercial operations in the Yuma area were followed except, in order to increase both the incidence and the homogeneity of aflatoxin contamination, insecticidal sprays to control the pink bollworm were not applied, as previously described (18,22). The test organism, AF36, was applied prior to first bloom (24 May 1989 and 13 June 1990) when the plants were 30–60 cm in height. The atoxigenic strain was distributed either by spraying plants (spray treatments) with a conidial suspension ( $2 \times 10^7$  spores/ml at a rate of 130 ml/m row length) or by spreading colonized wheat seed (wheat treatments) on the soil beneath the canopy at rates of 110 g and 8.4 g oven-dry weight per meter of row length in 1989 and 1990, respectively. On 14 September 1989 and 25 October 1990, approximately 2 kg of the mature crop per treatment per replicate was harvested by hand from a continuous segment of the treated area. All bolls on each plant were harvested, dried in a forced-air oven at 60 °C for 3 days, and stored in sealed plastic bags at room temperature until analyzed.

Sorting and quantification of locules infected prior to maturation. The percentage of the crop infected prior to maturation was based on the percentage (by weight) of locules (there were three to five locules per boll) with BG/YF (14). To reduce variability among determinations of aflatoxin content, the aflatoxin contents of locules with BG/YF and locules without BG/YF were determined separately (18). In 1989, seeds from the BG/YF locules were delinted with a small laboratory gin and sound seeds exhibiting BG/YF on the linters (small hairs not removed by ginning) were removed and divided into two portions, one for aflatoxin analyses and one for determination of the incidence of AF36. In 1990 there was a very low incidence of BG/YF locules due to low pink bollworm damage. Therefore, BG/YF locules were not processed with a gin. Instead, a single sound seed was removed from each BG/YF locule for fungal isolations and the remainder of each locule was analyzed individually for aflatoxin content.

Aflatoxin content of the crop. In 1989, 25-g portions of whole, ginned cottonseed were pulverized and extracted as previously described (13,18). Seed was pulverized with a hammer and added to 200 ml of acetone and water (85:15). The mixture was shaken for 15 s, allowed to set overnight, and filtered through a number 4 Whatman filter paper. A 100-ml portion of the filtrate was mixed with 100 ml of an aqueous solution of 0.22 M Zn(CH<sub>3</sub>COO)<sub>2</sub> and 0.008 M AlCl<sub>3</sub>, allowed to set 1–2 h and filtered again. A 100-ml portion of the filtrate was added to a 250-ml separatory funnel; aflatoxin extraction and analysis were performed as described for culture filtrates. Cottonseed exhibiting BG/YF on the linters and cottonseed without BG/YF were analyzed separately. To reduce variability, two separate analyses (25 g of seed each) of the non-BG/YF seed were performed for each replicate of each treatment and the results were averaged to determine the value for that replicate. For the 1990 test, the same technique was used as for the 1989 test, except that infected whole locules

(minus the single seed used to isolate the infecting strain) were extracted individually.

Monitoring strain distribution. The incidence of the vegetative compatibility group (VCG) of AF36 was determined to infer the distribution of that strain. To determine which isolates belonged to the VCG of AF36, nitrate-nonutilizing (*nir*) mutants of each isolate were generated using modifications of the previously described techniques (5). These modifications yielded *nir* mutants of all isolates tested whereas the previous technique yielded mutants from only 88% of tested isolates. Most fungal isolates spontaneously sectorized into nitrate-nonutilizing auxotrophs within 30 days at 32 °C after being transferred to a well in the center of the modified selection medium (Czapek-Dox broth (Difco) with 25 g/L KClO<sub>3</sub>, 50 mg/L rose bengal and 20 g/L agar, pH 7.0). A few isolates had to be transferred to the selection medium as many as four times. Auxotrophic sectors were transferred from the modified selection medium to Czapek-Dox broth with 15 g/L KClO<sub>3</sub> and 20 g/L agar (pH 6.5, 7 days, 32 °C) in order to stabilize the mutants. Mutants were subsequently grown on 5/2 agar and stored in sterile water, as described above, until used in complementation tests. Assignment of isolates to the VCG of AF36 was made on the basis of complementation tests (4) between *nirAD*<sup>−</sup> (deficient in the structural gene for nitrate reductase) and *enx* (deficient in a molybdenum cofactor) tester mutants (characterized by the method of Cove)(23) of AF36 and an uncharacterized *nir* mutant of the isolate to be assigned. Due to difficulties caused by the conidial nature of *A. flavus* and the instability of some mutants, only one complementation test was performed on each plate. Three wells (3-mm-dia), 2 cm apart, were cut in a triangular pattern in the center of the medium (20–25 ml) contained in 9-cm plastic petri dishes. The complementation medium consisted of Czapek-Dox broth adjusted to pH 6.0 with 2 N HCl, solidified with 2% agar (Bacto-Agar, Difco) and supplemented after autoclaving with Nitsch and Nitsch vitamin solution (Sigma) at twice the recommended concentration. For each complementation test, one well each was seeded with the AF36 tester mutants and one isolate mutant. Complementation occurred within 10 days at 32 °C. A total of 544 and 166 isolates were assessed with this method for the 1989 and 1990 tests, respectively.

The isolates used in the vegetative compatibility tests were obtained as follows. In 1989, 10–12 isolates of *A. flavus* from each replicate-treatment (384 total) were obtained from ginned seed with BG/YF linters. In 1990, isolates were obtained from one seed each of 34 BG/YF locules (38 BG/YF locules were harvested and four did not yield an *A. flavus* isolate; 16 of the 34 were from wheat treatment replicate plots; 18 of the 34 were from control replicate plots). Seed was wetted with a few drops of 95% ethanol, delinted for 3 min in concentrated sulfuric acid, washed three times in deionized water (2 min each), surface sterilized with 95% ETOH for 3 min, plated on the modified rose bengal medium described under quantification of fungal populations and incubated at 32 °C 5–10 days. *A. flavus* colonies were transferred to 5/2 agar and stored in sterile water, as described under cultures and inoculum preparation (above), until used to generate *nir* mutants. In 1990, *A. flavus* strains resident on the surfaces of the mature crop were also isolated. Three isolates per treatment per replicate (48 total) were picked from the dilution plates used to quantify these populations (below). In both 1989 and 1990, strains resident in soils both prior to application of AF36 and after harvest of the crop were isolated (two isolates per replicate treatment) from dilution plates used to quantify these populations (see below). To remove bias from colony selection, discrete colonies closest to plate centers were chosen.

Quantification of fungal populations. Populations of *A. flavus* in the soil were enumerated both 1 day before to application of treatments and 1 day after harvest in 1989 and 1990. Soil samples (35–50 g) from the top 2 cm of soil beneath the canopy were taken from each treatment-replicate. Two samples (48 total, no soil samples were taken from second untreated control) were taken on each date in 1989 and one sample (16 total) was taken on each date in 1990. Soils were dry (powdery) at sampling and

were sealed dry in plastic vials at room temperature until assayed within 1 mo. Populations in samples were enumerated by dilution plating on a modification of the rose bengal medium of Bell and Crawford (7). The modified rose bengal medium contained the following per liter: 10.0 g of NaCl, 3.0 g of sucrose, 3.0 g of Na<sub>2</sub>SO<sub>4</sub>, 0.3 g of KH<sub>2</sub>PO<sub>4</sub>, 0.7 g of K<sub>2</sub>HPO<sub>4</sub>, 0.5 g of MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5 g of KCl, 0.7 mg of Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>·10H<sub>2</sub>O, 0.5 mg of (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O, 10.0 mg of Fe<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>·6H<sub>2</sub>O, 0.3 mg of CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.11 mg of MnSO<sub>4</sub>·H<sub>2</sub>O, 17.5 mg of ZnSO<sub>4</sub>·7H<sub>2</sub>O, 50 mg of chloramphenicol, 10 mg of dichloran, 25 mg of rose bengal, 50 mg of streptomycin sulfate, and 20 g of agar. In 1990, the quantity of *A. flavus* superficially associated with the mature crop at harvest was also determined. Samples of seed-cotton (25 g) without BGYP were placed in culture bottles (1 L) containing 250 ml of 0.01% Triton X-100. Subsequently the bottles were shaken vigorously 1 min, allowed to settle 5 min and shaken again. The quantity of *A. flavus* propagules suspended in the liquid was then determined by dilution plate technique on the modified rose bengal agar.

**Aflatoxin-producing phenotypes.** An estimate of the spectrum of aflatoxin-producing phenotypes among isolates infecting the 1989 crop was made by determining the ability of infecting isolates to produce aflatoxin in liquid fermentation. From each replicate block, ten isolates in the VCG of AF36 and 10 isolates not in this VCG (160 isolates total) were checked for aflatoxin production in the liquid medium of Adye and Mateles (1) with 3 g/L NH<sub>4</sub>SO<sub>4</sub> as the nitrogen source as previously described (21). For each isolate approximately 3.5 × 10<sup>6</sup> spores were added to a single Erlenmeyer flask (250 ml) containing 70 ml of medium. Flasks were incubated in the dark on an orbital shaker (150 rpm) for 5 days, after which time 70 ml of acetone was added to each flask to extract the aflatoxins from the mycelium. Culture filtrates containing 50% acetone (v/v) were filtered through number 4 Whatman filter paper. Fifty milliliters of filtrate was added with an equal volume of water to a 250-ml separatory funnel and the solution was extracted twice with 25 ml of methylene chloride. The methylene chloride extracts were filtered through 50 g of anhydrous sodium sulfate to remove residual water and the sodium sulfate was rinsed with an additional 25 ml of methylene chloride after filtration. The rinse and extracts were combined, then evaporated at room temperature, and the residual was dissolved in 4 ml of methylene chloride. Extracts and aflatoxin standards (aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub>) were separated on TLC plates (silica gel 60, 250 mm) by development with diethyl ether-methanol-water (96:3:1) (39). Extracts were either concentrated or diluted to permit accurate densitometry (34) and aflatoxin B<sub>1</sub> was quantified with a scanning densitometer after development (model es-930, Shimadzu

Scientific Instruments, Inc., Tokyo) (34). The limit of detection was 1 µg/kg culture medium.

**Statistical analyses.** Analyses were performed with the Statistical Analysis System (SAS Institute, Inc., Cary, NC) and Microsoft Excel. ANOVA was used to test differences among treatments prior to application of multiple comparison techniques.

## RESULTS

**Incidence of BGYP.** In 1989, there was a great deal of pink bollworm damage (over 30% of the bolls were infested) and subsequent infection of developing bolls by *A. flavus* resulted in a high percentage of locules (22 ± 2% by weight; Table 1) that were positive for BGYP. In 1990, there was little pink bollworm damage (less than 5% of bolls were infested) and there were relatively few locules with BGYP (0.9 ± 0.1%). In both the 1989 and 1990 tests, the percentage of locules infected prior to boll maturity (BGYP locules) did not differ significantly (*P* = 0.05) among treatments.

**Aflatoxin content of the crop.** In both years, BGYP seed from plots treated with colonized wheat seed contained significantly less aflatoxin B<sub>1</sub> than BGYP seed from untreated control plots (Table 1). The aflatoxin B<sub>1</sub> content of the BGYP seed was 75–82% lower than the controls in 1989 and 99.6% lower in 1990. In 1989, the quantity of toxin in the seed not exhibiting BGYP was also determined. Only 2.6% of the detected aflatoxin occurred in seed not exhibiting BGYP and the quantity did not differ significantly among treatments.

The quantity of aflatoxin B<sub>1</sub> in the BGYP seed from the 1989 crop was inversely correlated with the percentage of isolates from that seed belonging to the applied VCG (Fig. 3). Replicate blocks containing high incidences of the applied VCG had low aflatoxin content and vice versa. Complete analyses were successfully performed on a total of 34 locules exhibiting BGYP on the lint in 1990. Only one of 18 locules from which an isolate belonging to the AF36 VCG was isolated contained detectable quantities of aflatoxins (Fig. 4). However, aflatoxin was detected in 13 of 16 locules (81%) from which an isolate not belonging to the applied VCG (the AF36 VCG) was isolated. Locules from which the applied VCG was isolated contained significantly (*P* = 0.05 by Student's *t*-test) less aflatoxin than locules from which other VCGs were isolated (0.2 µg/g versus 65.9 µg/g). Most locules (63%) from which other VCGs were isolated contained over 10 µg/g (Fig. 4).

**Strain distribution.** Nitrate-nonutilizing mutants were generated for all isolates examined (710 total). Prior to application of AF36, the incidence of its VCG in test field soils was one

TABLE 1. Influence of toxigenic *Aspergillus flavus* AF36 on incidence of bright-green-yellow-fluorescence (BGYP), aflatoxin content of harvested seed cotton, and incidence of *A. flavus* strains infecting and resident on surface of crop

Treatment <sup>1</sup>	BGYF (%) <sup>2</sup>		Aflatoxin B <sub>1</sub> (µg/g) <sup>3</sup>			Isolates in applied VCG (%) <sup>4</sup>			Quantity of <i>A. flavus</i> <sup>5</sup> on harvested crop (propagules/g)
	1989	1990	1989	1990	1990	Infecting isolates <sup>6</sup>		Surface isolates <sup>7</sup>	
			BGYF			Non-BGYF	1989		
Control 1	22 a <sup>8</sup>	0.85 a	39.0 a	0.7 a	81.8 a	25 c	7 b	4 b	28,059 a
Control 2	24 a	ND	53.5 a	0.8 a	ND	25 c	ND	ND	ND
Wheat	25 a	1.03 a	9.7 b	0.5 a	0.3 b	67 a	100 a	75 a	23,949 a
Spray	20 a	ND	36.8 a	1.6 a	ND	45 b	ND	ND	ND

<sup>1</sup> Control 1 = untreated control in the same row as treated plots; Control 2 = untreated control separated from the row with treated plots by four untreated rows; Wheat = application of colonized wheat to the soil beneath the canopy (110 g and 8.4 g dry weight per meter row length in 1989 and 1990, respectively); Spray = canopy sprayed with 130 ml per meter of 2 × 10<sup>7</sup> spores/ml in 0.01% Triton X-100.

<sup>2</sup> Percent seed-cotton exhibiting BGYP on a weight basis. Entire locules of seed-cotton were sorted into the BGYP category. Locules exhibiting even small amounts of BGYP were considered positive.

<sup>3</sup> Aflatoxin content is expressed per gram whole seed. Content of cotton exhibiting BGYP and cotton not exhibiting BGYP were determined separately.

<sup>4</sup> Percent isolates assigned to the applied vegetative compatibility group (VCG) to which AF36 belongs, on the basis of auxotroph complementation. Two isolates per replicate per treatment were assessed. ND = not determined.

<sup>5</sup> Quantity of *A. flavus* propagules washed from seed-cotton with 0.01% Triton X-100.

<sup>6</sup> Isolates from internal seed isolations.

<sup>7</sup> Isolates from surface washes of seed-cotton. Three isolates per replicate per treatment (48 total) were assessed.

<sup>8</sup> Values are averages of eight replicates. Values followed by the same letter are significantly different by Fisher's protected LSD test. ND = not determined.

of 48 isolates in 1989 and one of 36 isolates in 1990 (Table 2). By contrast, the overall frequency of the AF36 VCG within *A. flavus* soil populations increased by harvest ( $P = 0.05$  by the paired *t*-test) to 42 and 63% in 1989 and 1990, respectively (Table 2). However, differences ( $P = 0.05$  by analysis of variance) in the incidence of the AF36 VCG did not occur among treatments in either year and the VCG occurred in untreated control plots at a rate of 19 and 56% in 1989 and 1990, respectively (Table 2). After harvest, in 1989 the incidence of the applied VCG increased with distance ( $R^2 = 0.77$ ,  $P < 0.01$ ) from the south border of the test field (Fig. 5). A skewed pattern of distribution was not evident at harvest in 1990.

The applied VCG was also a major component of the *A. flavus* population infecting the crop during boll maturation (identified by BGYP) (2,18) (Table 1). Although the applied VCG was isolated from a greater percentage of the infected bolls from treated plots than from infected bolls from untreated controls, the applied VCG

was isolated from portions (25 and 7% in 1989 and 1990, respectively) of infected bolls from untreated plots in both years (Table 1). In 1989, the colonized wheat seed treatment resulted in the greatest level of the applied VCG in the infecting population (67 vs. 45% for the spray treatment) and, therefore, in 1990 only the colonized wheat seed treatment was used. In 1990, the applied VCG was isolated from all bolls exhibiting BGYP and harvested from the plots treated with colonized wheat seed. The incidence of the applied VCG within populations of *A. flavus* resident on the surfaces of seed-cotton at harvest was also determined in 1990. Seventy-five percent of isolates from seed-cotton surfaces from plots treated with colonized wheat seed in 1990 were assigned

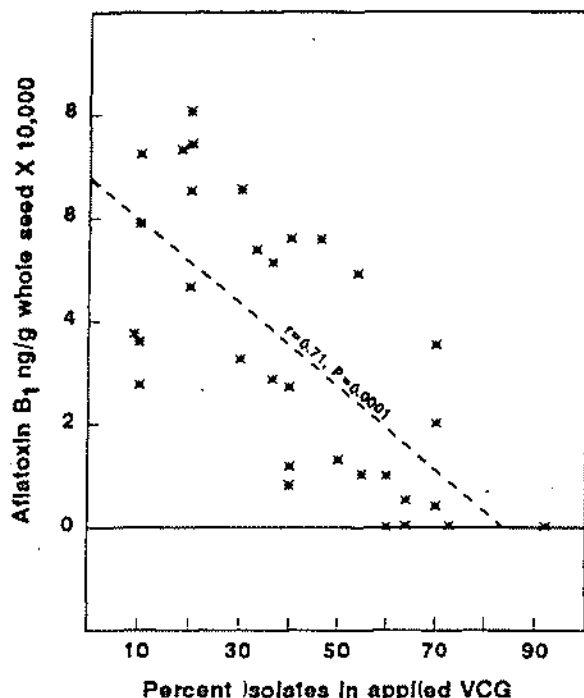


Fig. 3. Correlation of the incidence of the applied VCG among isolates internally infecting BGYP seed in 1989 and the quantity of aflatoxin  $B_1$  detected within that seed. Each point represents the average for a replicate plot (4 treatments  $\times$  8 replicates = 32 replicate plots).

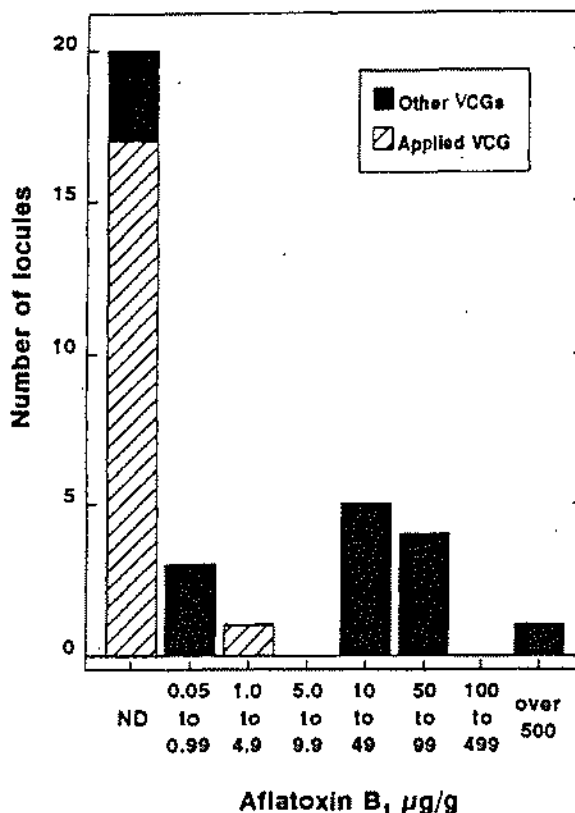


Fig. 4. Number of locules exhibiting BGYP and containing various concentrations of aflatoxin  $B_1$ . One *A. flavus* strain was isolated from one seed of each locule and determined to either belong to the applied atoxigenic VCG or not, through vegetative compatibility tests.

TABLE 2. Population density of *Aspergillus flavus* in soil and incidence of an applied vegetative compatibility group (VCG)

Treatment*	<i>A. flavus</i> (propagules/g)				Isolates in applied VCG (%) <sup>b</sup>			
	Before <sup>c</sup>		After <sup>c</sup>		Before		After	
	1989	1990	1989	1990	1989	1990	1989	1990
Control	2,979 <sup>d</sup>	1,100	4,288	11,038	6	6	19	56
Wheat	7,822	1,583	48,217*	55,858	0	0	69	69
Spray	5,596	ND	6,408	ND	0	ND	38	ND

\*Control = untreated control in the same row as treated plots; Wheat = application of colonized wheat to the soil beneath the canopy (170 g and 8.4 g dry weight per meter row length in 1989 and 1990, respectively); Spray = canopy sprayed with 130 ml per meter of  $2 \times 10^7$  spores/ml in 0.01% Triton X-100.

<sup>b</sup>Percent isolates assigned to the applied VCG on the basis of auxotroph complementation. Two to three isolates per replicate (16 total/replicate) were assessed. ND = not determined.

<sup>c</sup>'Before' samples were taken prior to first bloom on the day prior to treatment application (24 May 1989 or 13 June 1990) and 'After' samples were taken the day after harvest (14 September 1989 and 25 October 1990).

<sup>d</sup>Values are averages of 8 replicates. Overall before and after values differ significantly ( $P = 0.01$ ) for both 1989 and 1990 by the paired *t*-test. This holds for both *A. flavus* propagules/gram and for percent 'applied VCG'. The value denoted by "\*" differs significantly from other values in the same column ( $P = 0.05$ ) by Fisher's protected LSD test.

to the applied VCG on the basis of complementation tests, whereas only 7% of surface isolates from untreated plots were in the applied VCG.

**Magnitude of fungal populations.** The quantity of *A. flavus* on the surface of the seed at harvest was only quantified in 1990. High counts of propagules of *A. flavus* (over 20,000 per gram) were recovered from seed harvested from both treated and control plots in 1990 (Table 1). In 1989 and 1990, soil populations exceeded 1,000 propagules per gram prior to application of treatments and increased ( $P = 0.05$  by paired *t*-test) in all treatments between application and harvest (Table 2). Differences ( $P = 0.05$ ) among treatments were detected only in 1989 when soils from wheat treated plots had over 10-fold more propagules after harvest than did soils from the untreated control plots.

**Aflatoxin production by field isolates.** None of 80 isolates from the harvested seed belonging to the applied VCG produced detectable aflatoxin  $B_1$  levels in liquid fermentation. However, 80% of isolates not in the applied VCG produced detectable aflatoxin  $B_1$  and 65% of these isolates produced greater than 10  $\mu\text{g/g}$  of culture (Fig. 6).

## DISCUSSION

In 2 yr of field tests in Yuma, Arizona, soil application of atoxigenic *A. flavus* AF36 on colonized wheat seed resulted in a reduced quantity of aflatoxins in the cottonseed crop at maturity without an increase in the incidence of infection, as measured by BGJF. Vegetative compatibility analysis of fungal populations infecting the crops in both years provided evidence that these reductions were associated with displacement of the resident *A. flavus* population by the applied atoxigenic strain. In 1989, the atoxigenic strain was applied by spray, as well as on colonized wheat seed, but the spray application was not as effective as the colonized wheat seed in either displacement of the infecting population or prevention of the aflatoxin  $B_1$  accumulation in the infected portion of the crop. Greater efficacy of the colonized wheat seed treatment probably stems from a far greater quantity

of conidia being released for a longer period of time by the applied wheat than by the spray application.

The quantity of aflatoxin in plots treated with colonized wheat seed was 75–82% less than in untreated controls in 1989 and 99.6% less than in 1990. However, the applied VCG was isolated from 25 and 7% of infected seed in the untreated control plots in 1989 and 1990, respectively. Infection by AF36 or coinfection by AF36 and a strain not in the same VCG would be expected to result in lower aflatoxin levels than infection by most other VCGs alone [15,21]. Therefore, aflatoxin levels in control plots were probably lowered by atoxigenic strain applications, and the control of aflatoxin  $B_1$  contamination associated with the application of colonized wheat seed is probably underrepresented, especially for 1989. The correlation between incidence of the applied VCG and aflatoxin content of infected seed (Fig. 3) may better describe the impact of the atoxigenic strain on contamination.

Although the rate of application of wheat infested with the biocontrol agent in 1989 was greater than in 1990 (110 g/m row length versus 8.4 g/m row length), the percentage of the applied strain in infected locules from treated plots was only 67% in 1989 versus 100% in 1990. Lower displacement in treated plots in 1989 may have resulted from failure to treat rows adjacent to rows sampled at harvest; in 1990 rows on each side of the sampled rows were treated. Higher rates of displacement in 1990 with lower application rates more broadly dispersed may indicate that useful displacement and associated aflatoxin reductions can be achieved with much lower rates uniformly applied over larger contiguous areas.

The incidence of the applied VCG in infected seed from untreated control plots was much greater in 1989 than in 1990 (25 versus 7%). The crop was treated later with less material in 1990

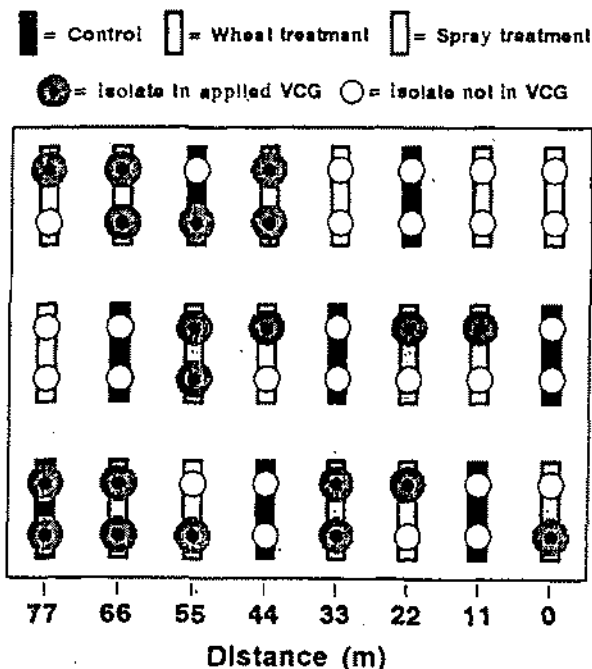


Fig. 5. Distribution of the applied vegetative compatibility group at harvest in 1989. Position of all 48 isolates is indicated. Predominant wind blew from right to left and the incidence of the applied strain increased with increased distance from the right border of the plot ( $R^2 = 0.77$ ,  $P < 0.01$ ).

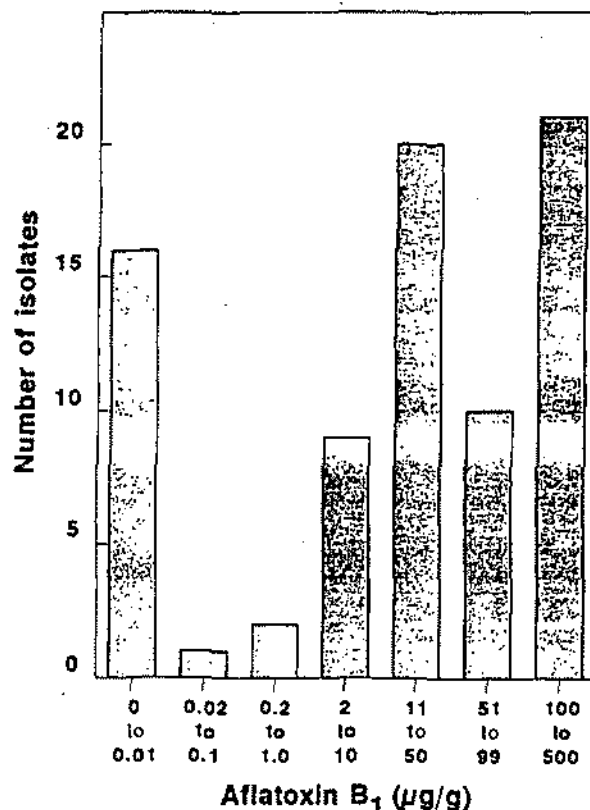


Fig. 6. Concentrations of aflatoxin  $B_1$  produced in liquid fermentation by isolates of *A. flavus* not in the applied VCG, from internal infections of seed exhibiting BGJF in 1989. The same quantity of isolates in the applied VCG were also tested but none of those isolates produced detectable quantities (0.01  $\mu\text{g/g}$ ) of aflatoxin  $B_1$ .

than in 1989 and reduced spread may have resulted from a combination of lower inoculum, a larger canopy at application, and environmental differences. It is surprising that in 1989, even though only 1.2% of the experimental field was treated with both spray and wheat treatments combined (if the amount of wheat applied had been dispersed over the entire plot, the application rate would have been 6.6 kg/ha), the average incidence of the applied strain was over 25% at the points most distant from applications.

There was a low incidence of the applied VCG infecting the developing crop (7%) and on the surfaces of the mature crop (4%) in untreated controls in 1990, but a high incidence in the soil of untreated plots after harvest. This differs from 1989 and a mechanism for this differential movement is unknown.

The rate of displacement by the applied VCG in both years suggests initial colonization of developing crops may greatly influence which fungal strains predominate during crop development. Introduction of new, uncolonized resources in the form of a crop uniformly developing may provide the opportunity for rapid swings in the composition of certain fungal populations associated with crops through colonization and establishment by relatively few initial strains. This phenomenon of epidemic increases in a few fungal types may occur frequently in agricultural fields. Such increases have been observed in unmodified *A. flavus* populations (4).

Strain application may increase the quantity of *A. flavus* inocula, at least initially. However, incidence of infection of developing cotton bolls did not differ between treated plots and untreated controls in either year. Predisposition of developing bolls (i.e., through insect activity) (22) may be a greater determinant of infection rate than the quantity of inoculum to which the crop is exposed. This may be particularly true in the desert valleys of Arizona where crops frequently are dusted by soil dispersed by agricultural activities and wind. This dust contains large quantities (at times exceeding 5,000 propagules/m<sup>3</sup> of air) (29) of *A. flavus* inocula. Furthermore, during the cotton season, very large proportions of dead and necrotic plant and animal tissue become colonized by *A. flavus* (3,38) and these contribute to inoculum levels. Thus, cotton bolls produced in these areas become exposed to large concentrations of *A. flavus* inocula.

Overall, *A. flavus* populations in the top 2 cm of the soil profile increased during the cotton season in both years (Table 2). These population increases occurred in both treated and control plots and the applied VCG composed significant portions (19 and 56%, in 1989 and 1990, respectively) of populations in control plot soils in both years. In 1989, the population in the soil of plots treated with colonized wheat contained significantly greater numbers of *A. flavus* propagules than untreated plots at harvest. Because the colonized wheat was delivered to the assayed sites 3 mo earlier, these differences might be expected. It is more surprising that in 1990 differences between treated and control plots were not significantly different. Nutrient sources other than the applied wheat must fuel *A. flavus* increases in these surface soils. Whether strain applications impact the quantity of *A. flavus* overwintering has not been determined. Overwintering populations may be determined to a greater extent by colonized organic matter than by the number of propagules resident at harvest.

The population of *A. flavus* on seed cotton surfaces at harvest in 1990 did not differ between treated and control plots and the applied VCG contributed only a minor portion (4%) of the propagules in the control plots and most (75%) of the propagules in the treated plots. It may, therefore, be possible to apply sufficiently low quantities of colonized matter to exclude resident strains without causing overall population increases. Exclusion apparently occurred during the *A. flavus* population increase that resulted in high propagule counts (over 20,000 propagules per gram) on the crop at harvest. Apparently the quantity of the fungus associated with the crop was dependent on a factor other than the quantity of fungus present early in the season when the colonized wheat was applied. Resources available for exploitation by this aggressive saprophyte and environmental conditions may dictate ultimate sizes of populations resident on the crops

to a greater extent than the magnitude of the initial fungal population, providing a certain minimal level of fungus is present. In both the 1989 and 1990 tests, over 1,000 propagules of *A. flavus* per gram of soil were present at the time of atoxigenic VCG application.

In 1990, aflatoxin was detected in one locule from which the applied VCG was isolated. Previous work has shown that many locules exhibiting BGYP are infected by multiple *A. flavus* strains (4) and that locules coinfected by toxigenic and atoxigenic strains contain less toxin (90–100% less) than locules infected by toxigenic strains alone (15,21). Analyses used in 1990 only permitted detection of a single infecting strain from each locule and that strain was isolated from a seed not used to determine the locule's aflatoxin content. The aflatoxin content of the locule in which both the applied VCG and aflatoxin were detected was probably attributable to infection by a second undetected strain and not to conversion of the atoxigenic strain to an aflatoxin-producing phenotype. Similarly, aflatoxin concentrations in locules either lacking detectable aflatoxin or with very low aflatoxin levels, but infected by strains other than the applied strain may be partially attributable to inhibition of toxigenesis by undetected coinfection by the applied strain. Possible conversion of atoxigenic strains to toxigenic strains after application has been suggested by several critics of the use of atoxigenic strains to prevent aflatoxin contamination (27). Aflatoxin-producing ability of certain *A. flavus* strains has been reported to be variable in culture (8,10) and certain strains apparently increase toxigenicity during boll infection (28). In the present study, no instability in atoxigenicity was detected among 80 isolates of the applied VCG from infected seed harvested 5 mo after strain application. This result, and the impact of applications on crop aflatoxin contents, suggest strain instability was not a problem during the course of experiments reported here. Furthermore, a recent study on the relationship of aflatoxin-producing ability to vegetative compatibility group suggested that aflatoxin-producing ability is relatively stable in individual strains and among groups of strains recently diverged (4). The authors further suggested that instability noted in previous studies might, at least in some cases, be attributable to in vitro culture methods.

The use of atoxigenic strains of *A. flavus* to prevent aflatoxin contamination is an unusual concept for the prevention of a plant disease problem. Like a few other biocontrol strategies (12,15), this strategy utilizes a strain of the species that incites the problem to be contained. However, unlike other strategies, for the atoxigenic strains to be effective during infection of the developing crop, the applied strains probably need to be at least as virulent as the strains they are directed at displacing. This may not be a requirement if strains are applied during periods in which the saprophytic habit of *A. flavus* is dominant. During such periods, atoxigenic strains with reduced virulence might be able to displace more virulent toxigenic strains during saprophytic utilization of crop and insect debris and thus reduce the incidence of highly virulent toxigenic strains.

Regardless of the means of intervention employed, there will be fungi associated with our crops. Dead, weakened, and partially decayed plant tissues are readily available and it is not feasible to prevent utilization of these resources by fungi. A level of control over which fungi become associated with crops may be permitted by the seeding of select fungal strains into agricultural fields in a manner similar to the seeding of plants. Such strains may be selected for adaptation to the crop ecosystem, reduced quantities of traits detrimental to human activity, and increased traits considered beneficial. This process of fungal domestication may permit minimization of certain problems caused by fungi (i.e., mycotoxin contamination) and optimization of beneficial fungal traits (i.e., degradation of crop debris). This is most likely to succeed where an undesirable fungal trait (e.g., mycotoxin production) is not necessary for fungal growth and multiplication on the crop or other substrate. However, many plant pathogenic fungi have large saprophytic phases. Deliberate introduction, during rotations to nonsusceptibles, of isolates with reduced virulence but greater saprophytic competitiveness may permit reductions in pathogenic potential in a manner similar to that

in which the reductions in aflatoxin-producing potential were achieved in the current study.

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Competitive Exclusion of a Toxigenic Strain of *Aspergillus flavus* by an Atoxigenic Strain

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## ABSTRACT

Cotty, P. J., and Bayman, P. 1993. Competitive exclusion of a toxigenic strain of *Aspergillus flavus* by an atoxigenic strain. *Phytopathology* 83:1283-1287.

Several experiments were employed to test the role of competition in the ability of an atoxigenic strain of *Aspergillus flavus* to inhibit the aflatoxin contamination of developing cotton bolls. In initial tests, nitrate-nonutilizing mutants were used to follow seed infection by toxigenic and atoxigenic strains of *A. flavus* in coinoculated bolls. Competitive exclusion was found to contribute to the effect of the atoxigenic strain on contamination, but results suggested a second mechanism may also have been in effect. Aflatoxin contamination by the toxigenic strain was similarly inhibited by an atoxigenic strain in vivo and in liquid fermentation, and the atoxigenic strain was equally effective when applied at spore concentrations either equal to those of the toxigenic strain or one-half those of

the toxigenic strain. The atoxigenic strain reduced aflatoxin production in vitro when mycelial balls of the two strains were mixed after a 48-h fermentation period, which suggested that close intertwining of mycelia was not required and that aflatoxin biosynthesis could be interrupted even after initiation. The atoxigenic strain did not degrade aflatoxins in vitro, and both culture filtrates and mycelial extracts of the atoxigenic strain stimulated aflatoxin production by the toxigenic strain. The results suggest that the atoxigenic strain may interfere with the contamination process both by physically excluding the toxigenic strain during infection and by competing for nutrients required for aflatoxin biosynthesis.

Aflatoxins are toxic, carcinogenic fungal metabolites that occur in foods and feeds worldwide (25). Health concerns and regulations that limit the uses of contaminated commodities greatly influence the profitability of several important crops in the United States. (25). Aflatoxins are produced by *Aspergillus flavus* Link:Fr. and *A. parasiticus* Speare when these fungi infect and decay either developing or mature crops (17). Conventional methods for the prevention of contamination are not reliable on a commercial scale for any of the crops affected; this has resulted in several attempts to develop novel control methods (9). One such method is to displace toxigenic strains of *A. flavus* from agricultural fields with strains of *A. flavus* that do not produce aflatoxins (atoxigenic strains) (10). This strategy is possible because of the great diversity of phenotypes of *A. flavus* in agricultural fields and the common occurrence of atoxigenic strains (13,18). Furthermore, toxigenicity is apparently unrelated to a strain's ability to colonize and/or infect living or dead plant tissues (13). These observations led us to speculate that atoxigenic strains might be adapted to conditions that favor aflatoxin contamination, and therefore atoxigenic strains might be used to displace toxigenic strains (13,14). In theory, competitive exclusion of toxigenic strains from crops might reduce the overall toxigenicity of *A. flavus* populations and might even interfere with the contamination process on an infection-by-infection basis (8,10,14).

Aflatoxin contamination of cottonseed is severe in the desert valleys of Arizona and southern California, where most aflatoxin occurs in seed from bolls damaged by the pink bollworm (15). Several atoxigenic strains of *A. flavus* isolated from agricultural fields in Arizona can reduce the aflatoxin contamination of developing cotton bolls caused by toxigenic strains (14). In the previous experiments, the atoxigenic strains greatly reduced contamination when inoculated into developing bolls either prior to or simultaneously with toxigenic strains. In those studies, however, it was not clear whether the atoxigenic strains prevented contamination by physically excluding the toxigenic strains from infected tissues or by directly inhibiting toxigenesis. The studies described here

sought a better understanding of the mechanism through which atoxigenic strains reduce contamination of developing bolls.

## MATERIALS AND METHODS

**Fungal isolates and mutants.** *A. flavus* isolates AF36 and AF13 collected from the Yuma Valley of Arizona and previously shown to be pathogenic to cotton were used in all tests (13). AF36 is atoxigenic and reduces aflatoxin contamination caused by the highly toxigenic AF13 (14). AF36 and AF13 were previously shown to belong to different vegetative compatibility groups (3). Cultures were maintained at 31°C in the dark on 5/2 agar (5% V8 vegetable juice, 2% agar adjusted to pH 5.2 prior to autoclaving) (13). For long-term storage, plugs of sporulating cultures were submerged in sterile distilled water and maintained at 8°C (13).

Two different mutants of both AF13 and AF36 were isolated on Czapek-Dox (CD) agar supplemented with chlorate as previously described (2). Mutants were maintained in the same manner as the wild types and characterized by the method of Cove (16). One mutant of each isolate was deficient in the structural nitrate reductase gene, *niaD*<sup>-</sup> mutant, and the other was defective in the pathway specific regulatory gene, *niaA*.

**Greenhouse tests.** To determine isolate behavior, developing cotton bolls were inoculated through simulated exit holes of the pink bollworm as previously described (13). *Gossypium hirsutum* cv. Deltapine acala 90 was grown in 3-L pots containing a 50:50 mixture of Pro-mix (Premier Brands Inc., New Rochelle, NY) and sand. Plants were fertilized weekly with about 100 ml of 2,000 ppm 15-30-15 fertilizer. Flowers were dated at opening. Bolls 29-31 days old were wounded in a single locule with a cork borer (3 mm in diameter) and inoculated with 20 µl of an aqueous spore suspension of each isolate tested. Spore concentrations and number of bolls per treatment are indicated below for each experiment. After boll opening (about 45 days after flowering), bolls were picked, dried in a forced-air oven at 45°C for 3 days, and stored at room temperature in plastic bags containing silica gel desiccant.

To determine whether the AF13 *niaD*<sup>-</sup> mutant retained wild-type ability to infect and contaminate developing cottonseed with aflatoxins and whether the *niaD*<sup>-</sup> mutant of AF36 retained wild-

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type ability to interfere with contamination, wild types and mutants were compared in an initial test with six treatments and three replicates. In four of the treatments, each boll was inoculated with 10,000 spores of either wild types or mutants of either AF13 alone or AF36 alone. In two of the treatments, each boll was inoculated with 10,000 spores each of either mutants or wild types of both AF13 and AF36. After the initial test demonstrated that wild types and mutants behaved similarly, the following tests were performed with the *niaD*<sup>-</sup> mutants alone in order to assess competition between the two strains during boll infection. Bolls were inoculated with 5,000 spores of the *niaD*<sup>-</sup> mutants of isolate AF13 alone, isolate AF36 alone, or both isolates AF13 and AF36. Tests were randomized and replicated three times. Replicates consisted of two bolls each; the inoculated locule from one boll of each replicate was analyzed for aflatoxin content, and the inoculated locule from the second boll was used for fungal isolations. This test was performed three times.

In greenhouse tests used to compare *in vivo* activity with *in vitro* activity, bolls were inoculated with wild-type strains. In the four treatments, bolls were inoculated with 1) 10,000 spores of AF13, 2) 10,000 spores of AF36, 3) 10,000 spores each of both isolates, or 4) 10,000 spores of AF13 and 5,000 spores of AF36. Locules from this experiment were analyzed only for aflatoxin content. Treatments were randomized and replicated four times; each replicate consisted of a single inoculated boll, and the experiment was performed twice.

**Fungal isolations and isolate identification.** Isolations from seed were made to determine the relative success of AF36 and AF13 in infecting developing cottonseed in coinoculated locules. Seeds were manually separated from the long lint fibers, wetted with 50% ethanol, delinted, surface-sterilized in concentrated H<sub>2</sub>SO<sub>4</sub> for 2–5 min, rinsed twice in sterile water, and plated on CD agar amended with antibiotics (4). Five isolations were made from colonies growing from each of four seeds from each inoculated locule for a total of 20 isolations per locule. Isolates were then paired with *nirA*<sup>-</sup> mutants of both AF13 and AF36 on CD agar, and complementation reactions after 10 days at 31 °C were used to identify the isolate (3). The test was performed three times. In the first two tests, isolations were made from bolls inoculated with AF13 and AF36 alone and from bolls inoculated with both isolates. In the third test, isolations were made only from bolls inoculated with both isolates.

***In vitro* interactions.** Interactions of strains AF36 and AF13 *in vitro* were assessed in the liquid medium of Adye and Matales (1) with either 3 g/L of NH<sub>4</sub>SO<sub>4</sub> (NH<sub>4</sub> medium) or 3 g/L of NaNO<sub>3</sub> (NO<sub>3</sub> medium) as the sole nitrogen source as previously described (11). Erlenmeyer flasks (250-ml) containing 50 ml of either NO<sub>3</sub> medium or NH<sub>4</sub> medium were inoculated with either 15,000 spores of AF13 per milliliter, 15,000 spores of AF36 per milliliter, 15,000 spores of AF13 and 15,000 spores of AF36 per milliliter, or with 15,000 spores of AF13 and 7,500 spores of AF36 per milliliter. Flasks were incubated in the dark on an orbital shaker (150 rpm) for 4 days, after which 50 ml of acetone was added to each flask to lyse fungal cells and extract the aflatoxins from the mycelium.

To ascertain potential inhibitory effects of AF36 on growth of AF13, isolates were paired on 5/2 agar and on CD agar by the inoculation of wells (3 mm in diameter) in the agar. The wells were spaced 2 cm apart. Two wells on each plate were inoculated with either the same isolate or with different isolates. Plates were incubated at 31 °C and visually examined after 5 days for zones of inhibition.

To determine the influence of actively growing mycelia of AF36 on aflatoxin production by mycelia of AF13, flasks containing NO<sub>3</sub> medium were inoculated with each isolate individually and shake-incubated as described above. After incubation periods of 0, 5, 10, 24, and 48 h, the contents of a flask containing AF13 and another containing AF36 were combined and mixed by swirling (2 min). The contents were then divided into approximately 50 ml per flask and shake-incubated for the remainder of 5 days from the initial inoculation; i.e., cultures mixed after 24 h were incubated for an additional 4 days. Control flasks

inoculated with AF13 alone were incubated for 1, 2, or 5 days and included in the randomized experimental design. After the incubation period, fungal growth was stopped by the addition of 50 ml of acetone, and aflatoxin analyses were performed as described below. This test was performed three times and replicated four times; the four replicates resulted from two separate mixings of paired flasks each consisting of one flask with AF13 and one flask with AF36.

**Filtrates, mycelial extracts, and degradation.** To test the involvement of factors that interfere with aflatoxin biosynthesis, AF36 in NO<sub>3</sub> medium was shake-cultured as described above for 4 days at 31 °C; the filtrate was then recovered and substituted for the water component of the NO<sub>3</sub> and NH<sub>4</sub> media. The influence of mycelial extracts of AF36 on aflatoxin biosynthesis by AF13 was also tested. Mycelial balls (15–20 g) from 4-day-old NO<sub>3</sub> medium shake cultures were blended at low speed for 30 s in 100 ml of distilled water. The aqueous extract was then filter-sterilized and added to NO<sub>3</sub> medium at a 10% rate (v/v). To test for degradation of aflatoxin by AF36, filtrates from 4-day-old shake cultures of AF13 in NO<sub>3</sub> medium were substituted for the water component of NO<sub>3</sub> medium as described above. In all three experiments, the media were filter-sterilized, dispensed into 50-ml Erlenmeyer flasks (30 ml per flask), inoculated, and incubated as described. Mycelial extract and filtrate experiments were inoculated with approximately 5,000 spores of AF13 and incubated for 4–5 days. Aflatoxin degradation tests were inoculated with approximately 5,000 spores of AF36 and incubated for 7 days. The experiments were performed at least twice and contained two to four replicates.

**Aflatoxin analyses.** Culture filtrates containing 50% acetone (v/v) were filtered through #4 Whatman filter paper. Fifty milliliters of filtrate was added with an equal volume of water to a 250-ml separatory funnel, and the solution was extracted twice with 25 ml of methylene chloride. The methylene chloride extracts were filtered through 50 g of anhydrous sodium sulfate to remove residual water, and the sodium sulfate was rinsed with an additional 25 ml of methylene chloride after filtration. The rinse and extracts were combined, evaporated at room temperature, and the residual was dissolved in 4 ml of methylene chloride. The extracts and aflatoxin standards were separated on thin-layer chromatography plates (silica gel 60, 250 mm) by development with diethyl ether-methanol-water (96:3:1) (32). Extracts were either concentrated or diluted to permit accurate densitometry (27), and aflatoxin B<sub>1</sub> was quantified with a scanning densitometer (model es-930, Shimadzu Scientific Instruments, Inc., Tokyo) after development (27).

Infected cotton locules were extracted as previously described (12). Dried intact locules were hammered to pulverize the seed and added to 200 ml of acetone and water (85:15). The mixture was shaken for 15 s, allowed to set overnight, and filtered through a #4 filter paper. A 100-ml portion of the filtrate was mixed with 100 ml of an aqueous solution of 0.22 M Zn(CH<sub>3</sub>COO)<sub>2</sub> and 0.008 M AlCl<sub>3</sub>, allowed to set for 1–2 h, and filtered again. A 100-ml portion of the filtrate was added to a 250-ml separatory funnel, and aflatoxin extraction and analysis were performed as described for culture filtrates.

**Statistical analysis.** Analyses were performed with CSS:Statistica (Statsoft, Inc., Tulsa, OK) and Excel (Microsoft Corporation, Redmond, WA). Treatments were randomized within experiments. Analysis of variance was used to test differences among treatments prior to application of multiple comparison techniques. Comparisons of proportions were made with the Z test (29).

## RESULTS

**Toxin content of inoculated bolls.** Bolls inoculated with *niaD*<sup>-</sup> mutants of both AF13 and AF36 had 0–20% of the aflatoxin content of bolls inoculated with the *niaD*<sup>-</sup> mutant of AF13 alone (Table 1). The AF13 *niaD*<sup>-</sup> mutant retained wild-type ability to infect and contaminate developing cottonseed with aflatoxins, and the AF36 *niaD*<sup>-</sup> mutant retained wild-type ability to inhibit aflatoxin contamination. In three experiments, locules inoculated

with the AF36 *nioD*<sup>-</sup> mutant alone contained no detectable aflatoxins, and locules inoculated with the AF13 *nioD*<sup>-</sup> mutant alone had 75–440 µg of aflatoxin B<sub>1</sub> per gram. Locules inoculated with wild-type AF13 typically contained similar toxin levels (14). In the one head-to-head test performed, locules inoculated with the AF13 *nioD*<sup>-</sup> mutant alone contained 159 ± 39 µg/g, and locules inoculated with AF13 wild-type contained 176 ± 22 µg/g.

Seed isolations from inoculated bolls. Results of isolations from locules inoculated with both *nioD*<sup>-</sup> mutants varied among the three experiments. In test 1, 70% of all isolates were AF36, and AF36 alone was isolated from one locule (five isolates from each of four seeds) and from three of eight seeds from two other locules. Only one seed (8%) was infected by AF13 alone. One-third of the seeds from the three locules were infected with both AF13 and AF36 (Table 1). In test 2, AF36 alone was isolated from one locule and from three of four seeds in a second locule. However, AF13 alone was isolated from one locule. Data from test 2 is presented in Table 1 with and without the locule containing

only AF13 (explained further in Discussion). In test 3, both AF13 and AF36 were isolated from all three locules, although 80% of all isolates in test 3 were AF36. AF36 alone infected seven of 12 seeds (58%), AF13 alone infected one of 12 seeds (8%), and four of 12 seeds were infected by both isolates. When isolations were made from seeds produced in locules inoculated with only one mutant, only that mutant was recovered.

Mutants of AF13 remained stable throughout the tests. However, in two of the *in vivo* tests, the *nioD*<sup>-</sup> mutant of strain 36 partially reverted to wild type in all bolls sampled. These revertants exhibited growth on CD agar intermediate between that of the wild type and the mutant. However, the growth remained sparse enough to permit detection of complementation between the revertant and the *nirA*<sup>-</sup> tester mutant.

*In vivo* versus *in vitro* activity. At maturity, locules inoculated with AF13 alone contained aflatoxin B<sub>1</sub> levels above 50 µg/g in all experiments. Locules inoculated with both AF13 and AF36 contained less than 10% of the aflatoxin in locules inoculated with AF13 alone. However, the quantity of toxin did not differ significantly ( $P = 0.05$  according to Fisher's protected least significant difference test) between locules inoculated with equal quantities of AF13 and AF36 and those inoculated with twice as much AF13 as AF36 (Table 2). Similar results were obtained in liquid fermentation in both NO<sub>3</sub> and NH<sub>4</sub> media. High toxin levels were produced in fermentations inoculated with AF13 alone, and low toxin levels occurred in fermentations inoculated with AF13 and AF36 together. The quantity of toxin in fermentations inoculated with twice as much AF13 as AF36 was equal to the toxin content of fermentations inoculated with equal quantities of AF13 and AF36.

On both CD and 5/2 agar media, mycelia of each strain grew until it met the mycelia of the other strain. The strains did not overgrow each other, and no zones of inhibition formed. Sporulation and mycelia were less dense where the colonies met, but this also occurred in self-confrontations.

When AF36 and AF13 were fermented individually, mixed after various periods, and re-fermented for the remainder of the 120-h test period, significantly less aflatoxin B<sub>1</sub> was produced by AF13 than in 120-h fermentations of AF13 alone (Table 3). In tests 1 and 3, linear regressions of aflatoxin content of mixed cultures with hours of growth until mixing were significant ( $P = 0.049$ – $0.026$ ). However, the  $R^2$  values were low ( $R^2 = 0.26$ – $0.34$ ). In tests 2 and 3, the aflatoxin content of cultures mixed after 48 h was significantly less than that of unmixed controls, even though aflatoxin production by AF13 had already begun at 48 h (Table 3).

Filtrates, mycelial extracts, and degradation. Culture filtrates of toxigenic AF13 that were filter-sterilized, supplemented with the nutrients of NO<sub>3</sub> medium, and incubated at 31 °C for 5 days did not have significantly different toxin levels than similar filtrates inoculated with AF36 prior to the incubation. All filtrates contained over 15 µg of aflatoxin B<sub>1</sub> per gram at the end of the experiments. Degradation of aflatoxin B<sub>1</sub> was thus not observed. Supplementation of NO<sub>3</sub> medium with either filtrates or

TABLE 1. Seed infected (%) with two strains of *Aspergillus flavus* at maturity after immature bolls were coinoculated with both strains and percent reduction of aflatoxin in bolls coinoculated compared to bolls inoculated with strain AF13 alone<sup>a</sup>

Test	Percent reduction <sup>b</sup>	Seed infected by inoculated strain(1) <sup>c</sup>			
		AF36 <sup>d</sup>	AF36 alone	AF13 alone	AF13 and AF36
1	82	70*	58*	8	33
2	100	64	58	33	8
2B <sup>e</sup>	100	96*	88*	0	13
3	99	80*	58*	8	33

<sup>a</sup>Toxin levels and seed infection were measured in parallel samples within each replicate.

<sup>b</sup>Percent reduction in aflatoxin B<sub>1</sub> content of seed from bolls coinoculated with atoxigenic strain AF36 and toxigenic strain AF13 compared to bolls inoculated with strain AF13 alone. Bolls inoculated with strain AF13 alone contained 76, 176, and 444 µg of aflatoxin B<sub>1</sub> per gram in tests 1, 2, and 3, respectively.

<sup>c</sup>Percentage of total isolates (55–60 isolates per test) from infected seed from locules inoculated with both AF13 and AF36 and identified as AF36. \* = Values significantly ( $P = 0.05$ ) greater than 50 by the Z test for proportions (29).

<sup>d</sup>At maturity, seeds were acid delinted, washed, and plated on selective medium; five isolations were made from each seed, and isolates were classified as AF36 or AF13 as described. Values represent the percentage of total seed infected by *A. flavus* that were infected by individual strains. There were 12 seeds per test, four seeds per replicate, five isolations per seed. \* = AF36 values significantly ( $P = 0.05$ ) greater than AF13 values by the Z test for proportions (29).

<sup>e</sup>In test 2, 57 isolates from 12 seeds from three bolls were evaluated; four seeds contained AF13 but not AF36. These all occurred in one boll (boll x) from which AF36 was not recovered. Test 2B is the data from test 2 minus boll x.

TABLE 2. Growth, toxin production, and alteration of culture pH by two strains of *Aspergillus flavus* grown individually and in combination

Strain ratio <sup>a</sup> (13:36)	Aflatoxin B <sub>1</sub>			Final pH <sup>b</sup>		Fungal mass (g)	
	In vivo <sup>c</sup>	NH <sub>4</sub> <sup>d</sup>	NO <sub>3</sub> <sup>d</sup>	NH <sub>4</sub>	NO <sub>3</sub>	NH <sub>4</sub>	NO <sub>3</sub>
1:0	501 x <sup>e</sup>	445 x	2.34 x	2.13 x	6.10 x	0.48 y	0.38 y
0:1	0 z	0 z	0 z	2.28 x	5.96 x	0.43 y	0.45 yx
1:1	8 y	30 y	3 y	2.30 x	5.71 x	0.50 xy	0.54 x
2:1	7 y	45 y	9 y	2.24 x	5.68 x	0.52 x	0.53 x

<sup>a</sup>Flasks containing 50 ml of medium were seeded with 16 conidia per microliter of either AF13 or AF36. For two treatments, flasks that were seeded with AF13 were also seeded with either 16 or eight conidia per microliter of AF36. Flasks were incubated at 28 °C for 4 days prior to being analyzed for aflatoxin content.

<sup>b</sup>Final pH of the culture medium; initial pH was 5.0.

<sup>c</sup>Cotton bolls 28–30 days old were inoculated with 10,000 conidia of either AF13 or AF36; plants in two treatments were inoculated with both AF13 and either 10,000 or 5,000 conidia of AF36. At maturity, seed from all inoculated bolls were analyzed for aflatoxin content, which is expressed as microgram per gram of whole seed.

<sup>d</sup>NH<sub>4</sub> = the NH<sub>4</sub> medium; NO<sub>3</sub> = the NO<sub>3</sub> medium. Aflatoxin is expressed as micrograms per gram of mycelium.

<sup>e</sup>Values for *in vivo* tests are averages of four replicates; values for *in vitro* tests are averages of three replicates. Values within a column followed by the same letter are not significantly different ( $P = 0.05$ ) according to Fisher's protected least significant difference test.

mycelial extracts of AF36 stimulated aflatoxin production by AF13 as compared to unsupplemented  $\text{NO}_3^-$  medium in every experiment. In one test, AF36 filtrates increased total aflatoxin  $\text{B}_1$  production nine-fold, and mycelial extracts increased production 68-fold (to 199  $\mu\text{g}$  per 70 ml fermentation), although in another test, toxin production was stimulated only four-fold by the mycelial extract.

## DISCUSSION

The *niaD*<sup>-</sup> mutants of AF36 and AF13 were useful in discerning strain prevalence during infection of developing cottonseeds in locules inoculated with both strains. Nitrate-nonutilizing mutants have previously been used to study plant pathogen population structure (3), to follow survival of *Fusarium oxysporum* in soil under field conditions (22), and to differentiate between phenotype switching and contaminants during host-passaging experiments with *Septoria nodorum* (26). Both the *Septoria* and *Fusarium* mutants retained virulence (22,26). Similarly, in the tests reported here, the *niaD*<sup>-</sup> nitrate-nonutilizing mutants retained virulence to developing cotton bolls. Furthermore, the *niaD*<sup>-</sup> mutant of AF13 retained the ability to contaminate developing cottonseed with aflatoxins, and the *niaD*<sup>-</sup> mutant of AF36 retained the ability to interfere with the process of contamination when coinoculated into developing cotton bolls. Similar mutants may have further uses in the investigation of interactions between strains in soils and on crop surfaces.

Although the *niaD*<sup>-</sup> mutant of AF36 was stable through 10 serial passages in culture, during infection of developing cotton bolls this mutant partially reverted to wild type in two of three experiments. Reversion did not occur with several strains of *Septoria nodorum* (26). The partially reverted AF36 mutants were still useful in complementation tests. However, reversion may have confused mutant identification as nitrate-nonutilizing if this was the sole criterion for identification. Thus, caution should be exercised when similar mutants of *A. flavus* are used to monitor strain migration in agricultural fields, as was done with *F. oxysporum* (22).

In all three boll inoculation tests, AF36 infected a greater percentage of the developing seeds than did AF13. Thus, the ability of AF36 to inhibit contamination by AF13 may partly be due to competitive exclusion of AF13 during infection of the develop-

ing cottonseed. In test 2, apparently one developing boll was inadvertently inoculated with only AF13; if AF13 had dominated in any of the locules analyzed for toxin, aflatoxin  $\text{B}_1$  would have been detected. The data for test 2 without the outlier boll (the boll infected with AF13 alone) probably better represents the true situation. Although competitive exclusion apparently is one mechanism through which AF36 reduces boll contamination, a second mechanism may also occur. This is suggested by the percent reduction in aflatoxin content that results from simultaneous inoculation with AF36 exceeding the percentage of total isolates identified as AF36 in each test (Table 1).

A second mechanism of action is also suggested by the ability of AF36 to interfere equally with contamination of developing cotton bolls by AF13 when either coinoculated at equal spore concentrations or at one-half the spore concentration of AF13. This phenomenon also occurred in liquid fermentation, and yet on solidified agar media at the same temperature as the fermentations, AF13 and AF36 did not appear to inhibit the growth of each other. Similar inhibition of aflatoxin production in liquid fermentation was observed when *A. parasiticus* was cofermented with *A. parasiticus* mutants blocked at specific steps in the aflatoxin biosynthetic pathway (20). Such mutants accumulate intermediates in the biosynthetic pathway and still exert a negative influence on aflatoxin biosynthesis without interfering with wild-type growth (21). In those studies, the influence of the mutants was not attributable to the mutants merely outgrowing the wild types. In experiments reported here, we tested whether the influence of AF36 on aflatoxin production was attributable to either degradation of aflatoxins, production of compounds inhibitory to aflatoxin biosynthesis, or competition for nutrients required for aflatoxin biosynthesis.

Degradation of aflatoxin by several fungi has been demonstrated, and even some strains of *A. flavus* and *A. parasiticus* partially degrade aflatoxin  $\text{B}_1$  after biosynthesis has stopped (5,7). In the current studies, AF36 did not degrade aflatoxin  $\text{B}_1$ . Degradation experiments were performed in medium with  $\text{NO}_3^-$  as the sole nitrogen source to prevent degradative effects attributable to low pH (11). Degradation is apparently not a mechanism through which AF36 acts. These observations are not necessarily in conflict with previous observations of degradation of aflatoxins by *A. flavus*, because the ability of a strain to degrade aflatoxin is thought to be correlated with strain ability to produce aflatoxins; atoxigenic strains are thought to have little ability to degrade aflatoxins (19).

AF36 exerted influence on aflatoxin production by AF13 even when AF13 mycelium was actively growing prior to exposure to AF36. Significant reductions occurred even when AF13 had initiated aflatoxin biosynthesis prior to exposure. Thus, inhibition of aflatoxin biosynthesis by AF36 differs from inhibition caused by both fungistatic and fungicidal agents that also inhibit growth; these chemical inhibitors are ineffective after initiation of aflatoxin biosynthesis (6,24,32). When spores are mixed in shake fermentation, the resulting mycelial balls are agglomerations of numerous germings. Thus, mycelia of the two strains become closely intertwined. Cultures mixed after 12 h have already formed mycelial balls. Inhibition in these mixtures indicates that mycelial intertwining is not required for activity of AF36. Similarly, intertwining of mycelium was not required for *A. niger* interference with aflatoxin production (31). These observations, taken together, suggest diffusible factors may be involved in the inhibitory ability of AF36.

Many bacteria and fungi can interfere with aflatoxin production (21,24,30). However, microbes that are as effective as AF36 are rare. Reduction in aflatoxin production in corn substrates coinoculated with *A. niger* and *A. flavus* have been attributed to alterations in substrate pH (23); no significant influence of AF36 on pH was observed in any of the tests reported here, suggesting alteration in pH is not a significant mechanism. Similarly, Shantha and coworkers found that pH was not involved in inhibition of aflatoxin production by *A. niger* and *A. tamarii* in liquid fermentation (31). These workers provided evidence for chemical inhibitors of aflatoxin biosynthesis. Results of the current study lead us to conclude that inhibition caused by AF36 probably does not

TABLE 3. Influence of atoxigenic strain AF36 on toxin production by toxigenic strain AF13 when strains are cultured separately for 0-48 h prior to mixing<sup>a</sup>

Treatment	Aflatoxin $\text{B}_1$ <sup>b</sup> ( $\mu\text{g/g}$ of mycelium)		
	Test 1	Test 2	Test 3
Mix asept			
0 h	1.4	8.5	5.2
5 h	2.4	11.4	0.5
10 h	17.7	28.8	2.7
24 h	ND	10.4	22.8
48 h	ND	5.0	30.8
AF13 control <sup>c</sup>	76.0	64.2	138.5

<sup>a</sup>Flasks containing 70 ml of  $\text{NO}_3^-$  medium were inoculated with either AF13 or AF36. After various periods, media containing AF36 were mixed with equal quantities of media containing AF13. The mixed contents were then divided into equal portions and returned to the original number of flasks. The resulting flasks were shake-incubated for the remainder of the 120-h incubation period and analyzed for aflatoxin content.

<sup>b</sup>Values are means of four replicates. Linear regressions (aflatoxin content of mixed cultures vs. hours growth until mixing) were significant for tests 1 and 3 ( $R^2 = 0.34$ ,  $P = 0.049$  and  $R^2 = 0.26$ ,  $P = 0.026$ , respectively) but not for test 2. For each test, all mix treatments differed significantly ( $P = 0.05$ ) from the unmixed controls according to Fisher's protected least significant difference test. ND = not determined.

<sup>c</sup>Control flasks were incubated with AF13 alone for 120 h. In tests 2 and 3, flasks with AF13 alone were also analyzed after 24 and 48 h. In both cases, no toxin was detected after 24 h and between 1 and 2  $\mu\text{g/g}$  of mycelium was detected after 48 h.

involve inhibitors. This conclusion is supported by stimulation of aflatoxin production by both culture filtrates and mycelial extracts of AF36. These observations do not rule out the possibility that short-lived inhibitors, i.e., the volatiles described by Zeringue and McCormick (33), might play a role. However, stimulation of toxigenesis by filtrates and mycelial extracts of AF36 suggests AF36 may reduce toxin production merely by competition for nutrients.

In one study of naturally infected cotton bolls, at least 50% were infected with multiple strains of *A. flavus* at maturity (3). In the current study, bolls inoculated at wounding with two strains often became predominantly infected by a single strain. Competition between strains initially infecting bolls may therefore cause an underestimation in the frequency of multiple infections. This competition may be an important determinant of the extent of contamination of naturally infected bolls. The ability of an atoxigenic strain to compete during colonization and infection of wounded locules may be a prerequisite for strain efficacy in the reduction of contamination in locules with multiple infecting strains.

Most studies on the physiology of aflatoxin formation have used only one isolate at a time. However, part of the possible range of phenotypes of a fungus may be expressed only when a mycelium confronts another genetic individual (28). The genotypic diversity of *A. flavus* in cotton fields and even in cotton locules ensures that competition between strains of *A. flavus* occurs in developing bolls. The data presented here show that this competition may have a complex effect on aflatoxin contamination and also suggests creative solutions for its control.

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Dr. Michael Braverman  
Technology Center of New Jersey  
Interegional Research Project No.4 (IR-4)  
681 U.S. Highway #1 South  
North Brunswick, NJ 08902-3390

Dear Dr. Braverman:

***Aspergillus flavus* AF36 (ai# 006456)**  
**Pending Section 3 Registration EPA Reg. No. 71693-R**  
**Pesticide Petition 8E5001**

A docket has been established for the pending Federal Register documents regarding the application for the Section 3 registration and exemption from tolerance associated for use of *Aspergillus flavus* AF36 on cotton in Arizona and Texas. We are requesting classification from IR-4, Dr. Peter Cotty and all relevant parties regarding the documents listed on the attached indexes. The classification categories are described as follows.

- A= Releasable to Anyone
- B= Releasable to persons who submit a signed Affirmation of Non-multinational Status form. If "B" documents appear on the index, a copy of the Affirmation is attached.
- C= Claimed Confidential by the submitter. Requests for "C" documents will be processed under the Freedom of Information Act and EPA's public information regulations at 40 CFR Part 2, subpart B.

Please fill out the attached form, so that we can quickly finalize these pending Federal Register notices that are essential for the registration of your product. Your assistance is appreciated. If you have any further questions regarding this matter, do not hesitate to email me or call at 703-308-8097.

Sincerely,

Shanaz Bacchus, Chemist  
Regulatory Action Leader  
Biopesticides and Pollution Prevention Division

Enclosure

1. Michael Braverman Biopesticide Coordinator  
(insert name and title)

of Interregional Research Project No.4 (IR-4) have classified the following documents  
pertaining to the Active ingredient *Aspergillus flavus* AF36 as indicated in the attached  
tables.

Signature/title/date Michael Braverman 12/20/02

Biopesticide Coordinator  
IR-4 Project

Send completed form to:

ATTN: Shanaz Bacchus (7511C)  
Biopesticides Pollution Prevention Division  
U.S. Environmental Protection Agency  
Ariel Rios Building  
1200 Pennsylvania Ave., N.W.  
Washington, DC 20460

Page 2 of 9

# Index of Documents Submitted

(As of February 8, 2002)

OPP- (Docket #)

**Aspergillus flavus AF36; Pending Section 3 Registration 71693-R; Establishment of Permanent tolerance exemption PP# 8E5001**

TITLE: (Title of document)	Author: (Last name, First name)	Document Date	Classification
1. Bibliography (attached)	EPA		
2. Application for Section 3 Registration for <i>Aspergillus flavus</i> AF36 for use on cotton in Arizona (AZ), Texas (TX).  Form 8570-1	Antilla, Larry	8/8/02	B
3. Transmittal Letter and attachments	Braverman, Michael	8/12/02	B
4. Risk Assessment of <i>Aspergillus</i> <i>flavus</i> AF36 (Federal Register Notice of Filing)	EPA Federal Register Document	TBD	A
5. Petition for a permanent exemption from the requirement of a tolerance for residues of products containing the active ingredient <i>Aspergillus flavus</i> AF36 on cotton (PP8E5001)	Braverman, Michael	8/12/02	C
6. Specific References to Supporting Data for the pesticide petition from IR-4 for <i>Aspergillus flavus</i> AF36, and the pending Section 3 registration for (EPA File Symbol 71693-R; PP8E5001; OPP Identifier Number(s) ) See references below.	See attached bibliography	See attached bibliography	C

Michael Braverman 12/20/02

TITLE: (Title of document)	Classification
43763400 USDA/ARS and IR-4 (1995) Submission of Product Chemistry, Toxicity, and Risk Data in Support of an Experimental Use Permit for <i>Aspergillus flavus</i> AF36. Transmittal of 5 Studies. SUBMITTED IN SUPPORT OF: 069224EX1 SUBMITTED IN SUPPORT OF: 5E4575	C
43763401 Cotty, P. (1995) <i>Aspergillus flavus</i> Isolate AF36--Product Identity and Disclosure of Ingredients, Manufacturing Process and Discussion on the Formation of Unintentional Ingredients: Lab Project Number: PR 52B. Unpublished study prepared by USDA/ARS. 85 p. SUBMITTED IN SUPPORT OF: 069224EX1 SUBMITTED IN SUPPORT OF: 5E4575	C
43763402 Cotty, P. (1995) <i>Aspergillus flavus</i> Isolate AF36--Analysis of Samples, Certification of Ingredient Limits, Analytical Methods for Certified Limits, and Physical and Chemical Properties: Lab Project Number: PR 52B. Unpublished study prepared by USDA/ARS. 8 p. SUBMITTED IN SUPPORT OF: 069224EX1 SUBMITTED IN SUPPORT OF: 5E4575	C
43763403 Cotty, P.; Hartman, C. (1995) <i>Aspergillus flavus</i> Isolate AF36--Safety Data in Support of Petition Proposing a Temporary Exemption from the Requirements of a Tolerance for <i>Aspergillus flavus</i> for Use in Cotton Production: Lab Project Number: PR 52B. Unpublished study prepared by USDA/ARS and IR-4. 882 p. SUBMITTED IN SUPPORT OF: 069224EX1 SUBMITTED IN SUPPORT OF: 5E4575	C
43763404 Cotty, P. (1995) <i>Aspergillus flavus</i> Isolate AF36: Hypersensitivity Incidents with Microbial Pest Control Agents: Statement of Finding No Hypersensitivity: Lab Project Number: PR 52B. Unpublished study prepared by USDA/ARS. 4 p. SUBMITTED IN SUPPORT OF: 069224EX1 SUBMITTED IN SUPPORT OF: 5E4575	C
43763405 Cotty, P.; Hartman, C. (1995) <i>Aspergillus flavus</i> Isolate AF36: Product Performance Data: Lab Project Number: PR 52B. Unpublished study prepared by USDA/ARS and IR-4. 145 p. SUBMITTED IN SUPPORT OF: 069224EX1 SUBMITTED IN SUPPORT OF: 5E4575	C

*Michael Braverman 12/20/02*

TITLE: (Title of document)	Classification
43972400 Interregional Research Project No. 4 (1996) Submission of Product Analysis and Toxicology Data in Support of an Experimental Use Permit for <i>Aspergillus flavus</i> AF36. Transmittal of 3 Studies. SUBMITTED IN SUPPORT OF: 069224EX1	C
43972401 Cotty, P. (1996) <i>Aspergillus flavus</i> Isolate AF36--Analysis of Samples, Certification of Ingredient Limits, Analytical Methods for Certified Limits: Amendment No. 1 to MRID No. 43763404: Lab Project Number: PR 52B: 52B. Unpublished study prepared by Southern Regional Research Center, USDA/ARS. 6 p. SUBMITTED IN SUPPORT OF: 069224EX1	C
43972402 Cotty, P. (1996) <i>Aspergillus flavus</i> Isolate AF36: Hypersensitivity Incidents with Microbial Pest Control Agents: Statement of Finding of No Hypersensitivity: Amendment No. 1 to MRID No. 43763404: Lab Project Number: 52B: PR 52B. Unpublished study prepared by Southern Regional Research Center, USDA/ARS. 4 p. SUBMITTED IN SUPPORT OF: 069224EX1	C
✓ 43972403 Shelton, L. (1996) Acute Oral Toxicity Study in Rats: ( <i>Aspergillus flavus</i> AF36): Final Report: Lab Project Number: M96AG84.6G31: MA M96AG84.6G31. Unpublished study prepared by Microbiological Associates, Inc. 59 p. SUBMITTED IN SUPPORT OF: 069224EX1	C
43990000 Interregional Research Project No. 4 (1996) Submission of Product Chemistry Data in Support of the Application for Experimental Use Permit for <i>Apergillus flavus</i> AF36. Transmittal of 1 Study. SUBMITTED IN SUPPORT OF: 069224EX1	C
43990001 Cotty, P. (1996) <i>Aspergillus flavus</i> Isolate AF36--Product Identity and Disclosure of Ingredients, Manufacturing Process, and Discussion on the Formation of Unintentional Ingredients: Amendment No. 1 to MRID 43763401: Lab Project Number: PR 52B. Unpublished study prepared by USDA/ARS, Southern Regional Research Center. 6 p. SUBMITTED IN SUPPORT OF: 069224EX1	C
44597000 Interregional Research Project No.4 (1998) Submission of Product Chemistry Data in Support of the Petition for Tolerance of <i>Aspergillus flavus</i> isolate AF36 in/on Wheat. Transmittal of 1 Study. SUBMITTED IN SUPPORT OF: 8E5001	C

TITLE: (Title of document)	Classification
<p>✓ 44597001 Cotty, P.; Antilla, L. (1998) Aspergillus flavus Isolate AF36 Manufacturing Process and Discussion on the Formation of Unintentional Ingredients. Amendment No. 2 MRID 43763401: Lab Project Number: 52B. Unpublished study prepared by USDA/ARS, Arizona Cotton Research and Protection Council and Rutgers Univ. 38 p. SUBMITTED IN SUPPORT OF: 8E5001</p>	C
<p>44626100 Interregional Research Project No. 4 (1998) Submission of Product Chemistry Data in Support of the Petition for Tolerance of Aspergillus flavus isolate AF36 in/on Cotton. Transmittal of 1 Study. SUBMITTED IN SUPPORT OF: 069224EX1 SUBMITTED IN SUPPORT OF: 5E4575</p>	C
<p>44626101 Cotty, P.; Antilla, L. (1998) Aspergillus flavus isolate AF36-Analysis of Samples, Certification of Ingredient Limits, Analytical Methods for Certified Limits: Amendment No. 2 to MRID No. 43763402: Lab Project Number: 52B. Unpublished study prepared by USDA/ARS, and Arizona Cotton Research and Protection Council. 33 p. SUBMITTED IN SUPPORT OF: 069224EX1 SUBMITTED IN SUPPORT OF: 5E4575</p>	C
<p>44713700 Interregional Research Project No.4 (1998) Submission of Product Chemistry Data in Support of the Petition for Tolerances of Aspergillus flavus in/on Cotton. Transmittal of 1 Study. SUBMITTED IN SUPPORT OF: 8E5001 SUBMITTED IN SUPPORT OF: 5E4575 SUBMITTED IN SUPPORT OF: 069224EX1</p>	C
<p>✓ 44713701 Cotty, P.; Antilla, L. (1998) Aspergillus Flavus isolate AF36--Amended Manufacturing Process--Amendment No.3: Lab Project Number: 52B. Unpublished study prepared by IR-4. 21 p. SUBMITTED IN SUPPORT OF: 8E5001 SUBMITTED IN SUPPORT OF: 5E4575 SUBMITTED IN SUPPORT OF: 069224EX1</p>	C

Michael Braverman 12/20/02

TITLE: (Title of document)	Classification
<p>45307200 USDA/ARS Southern Regional Research Center (2001) Submission of Environmental Fate Data in Support of the Petition for Tolerance of <i>Aspergillus flavus</i> Isolate AF36/Cotton in/on Cotton. Transmittal of 2 Studies.</p> <p>SUBMITTED IN SUPPORT OF: 5E4575 SUBMITTED IN SUPPORT OF: 069224EX1</p>	C
<p>45307201 Cotty, P. (2001) <i>Aspergillus flavus</i> Isolate AF36: Safety Information (Soil and Air Monitoring of Populations of <i>A. flavus</i>): Lab Project Number: 52B. Unpublished study prepared by Interregional Research Project No.4. 130 p.</p> <p>SUBMITTED IN SUPPORT OF: 5E4575 SUBMITTED IN SUPPORT OF: 069224EX1</p> <p>Start here</p>	C
<p>45739100 Interregional Research Project No. 4 (2002) Submission of Toxicity and Exposure Data in Support of the Petition for Tolerance of <i>Aspergillus flavus</i> on Cotton. Transmittal of 4 Studies.</p> <p>SUBMITTED IN SUPPORT OF: 2E6497</p> <p>45739103 Smith, D.; Cotty, P.; Braverman, M.; et al. (2002) <i>Aspergillus flavus</i> Isolate AF36: Non-Target Organism and Environmental Safety Information: Lab Project Number: IR-4 PR NO.52B: Unpublished study prepared by Soil &amp; Crop Sciences, Southern Regional Research Center USDA/ARS, Rutgers University and Arizona Cotton Research and Protection Council. 57 p.</p> <p>SUBMITTED IN SUPPORT OF: 8E5001</p>	C
<p>45739101 Blanchard, E.; Carter, J. (2002) <i>Aspergillus flavus</i> AF36: Acute Pulmonary Toxicity and Pathogenicity to the Rat: Interim Report: Lab Project Number: UAR/006. Unpublished study prepared by Huntingdon Life Sciences, Ltd. 86 p. {OPPTS 885.3150}</p> <p>SUBMITTED IN SUPPORT OF: 8E5001</p>	C

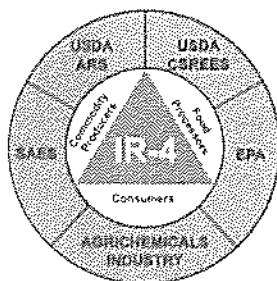
*Michael Braverman 12/20/02*

TITLE: (Title of document)	Classification
<p>45739104 Antilla, L.; Cotty, P.; Braverman, M. (2002) <i>Aspergillus flavus</i> Isolate AF336: Hypersensitivity Incidents: Lab Project Number: 52B. Unpublished study prepared by Arizona Cotton Research and Protection Council, Southern Regional Research Center and Rutgers University. 18 p.</p> <p>SUBMITTED IN SUPPORT OF: 8E5001</p>	C
<p>45739102 Mayer, D. (2001) Honey Bee Field Study of <i>Aspergillus flavus</i> AF36 in Cotton: Lab Project Number: WSU 00-011. Unpublished study prepared by Washington State University. 30 p. {OPPTS 850.3040, 885.4380}</p> <p>SUBMITTED IN SUPPORT OF: 8E5001</p>	C
<p>45307202 Cotty, P. (2001) <i>Aspergillus flavus</i> Isolate AF36 Non-target Organism and Environmental Safety Information (Soil and Air Monitoring of Populations of <i>A. flavus</i>): Lab Project Number: 52B. Unpublished study prepared by Interregional Research Project No.4. 130 p.</p> <p>SUBMITTED IN SUPPORT OF: 5E4575 SUBMITTED IN SUPPORT OF: 069224EX1</p>	C
<p>45798100 Southern Regional Research Center (2002) Submission of Toxicity Data in Support of the Registration of <i>Aspergillus flavus</i> AF-36 and the Petition for Tolerance of <i>Aspergillus flavus</i> isolate AF-36 in/on Cotton. Transmittal of 2 Studies.</p> <p>SUBMITTED IN SUPPORT OF: 8E5001</p>	C
<p>45798101 Blanchard, E. (2002) <i>Aspergillus flavus</i> AF36: Acute Pulmonary Toxicity and Pathogenicity to the Rat: Lab Project Number: UAR/004: UAR004/014519/AC: PR 52B. Unpublished study prepared by Huntingdon Life Sciences Ltd. 53 p. {OPPTS 885.3150}</p> <p>SUBMITTED IN SUPPORT OF: 8E5001</p>	C

*Michael Braverman 12/20/02*

TITLE: (Title of document)	Classification
<p>45798102 Rodgers, M. (2002) Toxicity/Pathogenicity to the Bobwhite Quail: Avian Inhalation Test Tier 1: Aspergillus flavus AF36: Lab Project Number: UAR 005: UAR 005/022336: PR 52B. Unpublished study prepared by Huntingdon Life Sciences Ltd. 21 p. {OPPTS 885.4100}</p> <p>SUBMITTED IN SUPPORT OF: 8E5001</p>	C
<p>45798200 Southern Regional Research Center (2002) Submission of Toxicity Data in Support of the Registration of Aspergillus flavus and the Petition for Tolerance of Aspergillus flavus Isolate AF36 in/on Cotton. Transmittal of 1 Study.</p> <p>SUBMITTED IN SUPPORT OF: 8E5001</p>	C
<p>45798201 Blanchard, E. (2002) Aspergillus flavus AF36: Acute Pulmonary Toxicity and Pathogenicity to the Rat: Lab Project Number: UAR/006: UAR 006/023279/AC. Unpublished study prepared by Huntingdon Life Sciences Ltd. 61 p. {OPPTS 885.3150}</p> <p>SUBMITTED IN SUPPORT OF: 8E5001</p>	C

*Michael Braverman 12/20/02*



**Interregional Research Project No. 4  
Center for Minor Crop Pest Management**

Shanaz Bacchus  
Biopesticide and Pollution Prevention Division  
Document Processing Desk  
Office of Pesticide Programs  
U.S. Environmental Protection Agency  
Second Floor, Crystal Mall 2  
1921 Jefferson Davis Highway  
Arlington, VA 22202-4501  
(703)308-8097

March 25, 2002

Dear Shanaz:

RE: *Aspergillus flavus* AF36

As you requested during our conversation on March 20, 2002 I have attached a summary of the toxicology research. Additional toxicology work has been submitted previously, therefore this summary only represents research not yet submitted to EPA. These will be submitted along with the Section 3 registration package.

There have not been any adverse affects attributable to *Aspergillus flavus* AF36 and the primary points of consideration for the continuation and expansion of the EUP and are as follows:

1. The honey bee study determined that *Aspergillus flavus* AF36 is considered non-hazardous.
2. There was no evidence of infectious risk in either avian or mammalian studies
3. There have been no reported adverse effects during the research or EUP phases of product production, development and evaluation.
4. *Aspergillus flavus* AF36 is already found in the soils of the states requested in the EUP.

Technology Centre of New Jersey  
681 U.S. Highway #1 South • North Brunswick, NJ 08902-3390 • 732/932-9575 • Fax: 732/932-8481

5. Aflatoxin is a known toxin and *Aspergillus flavus* AF36 has the ability to reduce this toxin.

Thank you for continued guidance in the EUP process. The EUP for Arizona and Texas will enable additional information to be collected so that EPA can make a better informed decision when the Section 3 registration package is submitted.

Thank you for your time in reviewing this information.

Sincerely,



Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
FAX (732)932-8481  
braverman@aesop.rutgers.edu

CC: Phil Hutton, Bob Holm, Peter Cotty

Enclosure- Toxicology Summary

## Preliminary Summary of Toxicology studies on *Aspergillus flavus* AF36

Michael Braverman, IR-4 Project

March 25, 2002

This is a preliminary summary of the results of toxicology studies of *Aspergillus flavus* AF36 in bees, birds and rats performed during 2001 and 2002. We are awaiting the reports with detailed information from the toxicology laboratory (Huntingdon Life Sciences, Ltd. Huntingdon, England). In addition to these studies, through the history of laboratory research, production of *A. flavus*-colonized wheat seed and in field use of this product under the current EUP, there have not been any reported ill effects. This has included manufacturing personnel, field and laboratory staff, and growers and field workers. Applications of *Aspergillus flavus* AF36 have been made to commercial fields since 1996 and a total of over 40,000 acres of commercial cotton in Arizona have been treated with *Aspergillus flavus* AF36. Over 400,000 pounds of wheat seed colonized by *Aspergillus flavus* AF36 has been produced at the manufacturing facility in Phoenix. This facility has been developed and built by a partnership between the Agricultural Research Service of the United States Department of Agriculture and the Arizona Cotton Research and Protection Council (ACRPC). The ACRPC is statutory agency of the State of Arizona and is run by a board of cotton producers appointed by the Governor of Arizona in consultation with the Arizona Cotton Growers Association.

Previously submitted information has documented that *Aspergillus flavus* is common on crop and native plants and in soils throughout the areas in which *Aspergillus flavus* AF36 will be applied. Furthermore, it has been documented that *Aspergillus flavus* AF36 is ubiquitous in the areas of Arizona and Texas for which Experimental Use Permits have been requested.

### Material for Toxicology Studies

For all studies *Aspergillus flavus* AF36 was produced in the same manner as when applied to commercial fields for reduction of aflatoxin producing fungi. For the initial mammalian study conidia were produced on sterile wheat seed in sterile bottles just prior to animal dosing. The conidia were washed from the wheat with rigorous shaking in 0.5% Tween 80. In subsequent studies, the avian study and the dose-range study in rat, the conidia were washed from the wheat in sterile physiological saline. For the bee study, colonized wheat seed was applied to a commercial cotton field in the routine manner.

### Honey Bee Study

A study was conducted by The Bee Group of Washington State University. AF-36 colonized wheat seed was applied aerially at 10 lb product/acre to a 40 acre cotton field near Eloy Arizona. This is the rate always used in treatment of commercial fields. A 40-acre control plot was also included. Twelve European Honeybee colonies in the plots were observed for the number of dead bees, number of foraging bees and number of frames of adult bees from 3 to 30 days after application for a total of 24 evaluations.

Researchers used a rating scale in which <100 dead bees/colony /day is considered normal die off (Non-hazardous). Less than 100 bees died (Maximum 86) in all

evaluations in the treated plot. Greater than 100 bees died (122 and 114) in the untreated plots during 2 of the 24 evaluations. There were significantly more dead bees in the treated plots on three of the ratings. There were significantly more dead bees in the untreated plot at one of the ratings. There was no significant difference in the number of bee foragers or frames of bees between the treated and untreated plot. The researchers concluded that AF36 is non-hazardous to bees and can be applied to cotton in bloom with minimal hazard.

#### Avian Inhalation Study

Study was conducted by Huntington Life Sciences, England. Thirty Bobwhite Quail received five mean daily doses of AF36 at  $3.75 \times 10^5$  cfu per bird by intratracheal instillation. Two groups of ten birds were allocated as controls: negative control (five undosed birds and five birds receiving the vehicle) and a heat-killed control.

There were no treatment-related mortalities. Observations over 35 days showed no clinical signs of toxicity and no treatment-related effects evident in either bodyweight change or food consumption. No abnormalities were observed at macroscopic post mortem examination. Treatment with *Aspergillus flavus* AF36 produced no toxicity and no infectivity.

#### Mammalian Studies

The initial pulmonary rat study which resulted in lethality in a significant number of animals treated with either the live *Aspergillus flavus* AF36 in Tween 80 or heat killed *Aspergillus flavus* AF36 in Tween 80. Onset of symptoms was rapid after dosing with all deaths occurring by day four of the study. All rats surviving to day four of the study recovered and all rats sacrificed (as scheduled) on day 8 or day 15 of the study had totally eliminated viable *Aspergillus flavus* AF36 from the lungs, ceecal contents, and faeces. There was no evidence of infectivity. The aetiology of deaths was unclear. However, it is possible that *Aspergillus flavus* AF36 prepared using Tween 80 caused a severe acute inflammatory response. Retrospective literature review and consultation with a toxicologist supported the theory that the responses were a result of a synergism with Tween 80 and/or of Tween 80 breakdown products formed during preparation of the spore suspension.

A second rat study was therefore undertaken. In the second study the conidia were both washed from the wheat and suspended in sterile physiological saline instead of Tween 80. Animals (2 male and 2 female for each treatment level) were dosed at 0,  $10^5$ ,  $10^6$ ,  $10^7$ , and  $10^8$  colony forming units per rat. There were no clinical signs in any of the treatment groups considered to be associated with the test substance. Rats were sacrificed at day 8 without treatment associated mortality. No abnormalities were observed in any of the animals at the macroscopic examination at termination.

Based on these two mammalian studies, we concluded that *Aspergillus flavus* AF36 does not present either a toxicological or an infectious risk to mammals.

United States Environmental Protection Agency

AF36  
mtg  
Helmut

AUG 14 2002

Dr. Michael Braverman, Coordinator  
Interregional Research Project No.4  
681 US Highway #1 South  
North New Brunswick, NJ 08902

Subject: Minutes of Meeting - Section 3 Registration: *Aspergillus flavus* AF36

Dear Dr. Braverman:

This letter responds to your letter of June 7, 2002 regarding the minutes of the meeting held here in Crystal City, May 30, 2002, to discuss the proposed Section 3 registration of *Aspergillus flavus* AF36.

The studies needed to complete the package are the mammalian toxicology studies to demonstrate the pulmonary effects of the active ingredient and the non-target avian pulmonary and honey bee study. Requests to waive data for other non-target organism or any other studies to fulfil ecological effects or other guideline requirements must be submitted in writing and be accompanied by sound scientific rationales.

When setting your goals for the Section 3 registration, please keep in mind that the approval depends on the timely submission of data as well as the acceptability of the submissions. If data reviews identify deficiencies, then supplementary data may be required to address those deficiencies. Such situations must be factored into your time line.

With regards to the label, you must consider the Agency's label requirements as outlined in the label review manual and the 40 CFR and modify the label accordingly.

You mention that you were not aware of aflatoxin reduction being a public health issue. In the Federal Register notice of February 14, 1996, (FR vol. 61, p. 5771) the Agency published the Notice of Receipt of the application for the Experimental Use Permit as SB:71693R:7302002:7511C

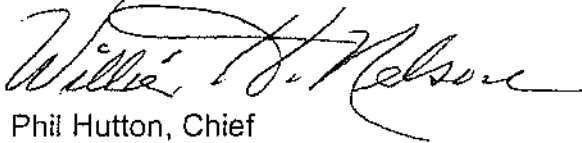
CONCURRENCES

SYMBOL ▶	7511C							
SURNAME	ETSI/TTI	TOMIYASU	VAITUZIS					
DATE	Aug 6, 02	Aug 6 2002	Aug 8, 02					

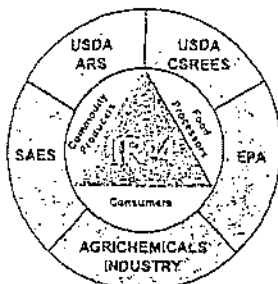
being of regional and national significance. Again on June 14, 1996, the discussion of the granting of the Experimental Use Permit (FR vol. 61 p. 30235) stated that the use of the microbial pesticide is intended to "protect public health". Efficacy data are required for all pesticides to mitigate public health hazards.

I trust that the foregoing clarifies the minutes of the meeting. If you have any additional questions regarding this registration, do not hesitate to email Shanaz Bacchus at [bacchus.shanaz@epa.gov](mailto:bacchus.shanaz@epa.gov) or call her on 703-308-8097.

Sincerely,

A handwritten signature in dark ink, appearing to read "William H. Nelson", is written over the typed name "Phil Hutton".

Phil Hutton, Chief  
Microbial Pesticides Branch  
Biopesticides and Pollution Prevention Division



**Interregional Research Project No. 4  
Center for Minor Crop Pest Management**

Dr. Janet Andersen  
Biopesticide and Pollution Prevention Division  
Environmental Protection Agency  
Room 910, Crystal Mall 2  
1921 Jefferson Davis Highway  
Arlington, Virginia

June 7, 2002

RE: *Aspergillus flavus* AF36 PC Code 006456  
Minutes of meeting- Section 3 Registration May 30, 2002

EPA Participants- Janet Andersen, Phil Hutton, Shanaz Bacchus, Gail Tomimatsu,  
Zigfridass Vaituzis, Carl Etsitty

Registrant Related Participants- Peter Cotty-USDA/ARS, Phil Wakelyn, Keith Menchey-  
National Cotton Council, Larry Antilla- Arizona Cotton Research and Protection Council,  
Chuck Youngker, Arizona Cotton Growers Association, Michael Braverman-IR-4 Project.

Dear Janet:

On behalf of the USDA/ARS, National Cotton Council, Arizona Cotton Council, Arizona Cotton Growers Association and the IR-4 Project, I would like to thank you and your staff for taking the time to meet with us to discuss the section 3 registration of *Aspergillus flavus* AF-36 on May 30, 2002. We were especially impressed with your interest and questions about the project and its importance to growers and public health. According to my records, the last Section 3 meeting was held about two years ago on June 27, 2000 (copy attached). It appears that from that previous meeting it was agreed that the studies needed to complete the registration package were the toxicology studies, which have now been completed.

*Chamunian + Montarget Aron + honey bee*

Technology Centre of New Jersey  
681 U.S. Highway #1 South • North Brunswick, NJ 08902-3390 • 732/932-9575 • Fax: 732/932-8481

\* For fulfillment of Ecological Effects submission  
of single species toxicity testing or a formal request to waive  
further testing must be submitted. All waiver requests must be  
accompanied by scientific rationale. Several data reviews  
(please refer to table) have addressed  
The most important highlights of the current meeting and our understanding of what is needed to  
complete the data requirements for Section 3 registration for AF-36 and the timetable for EPA  
review of these data are as follows:

When formally submitted (and assuming they are acceptable), the mammalian, avian and bee  
toxicology studies will essentially complete the data requirements for the section 3 registration.

Many parts of the current label language were developed due to the lack of toxicology data and  
if justified, can be modified.

The goal for section 3 registration is February 2003 so that we can avoid the inefficiency of  
having to request and review an additional expansion of the EUP. This depends on  
Submission of a complete package and acceptability of studies.  
The following are our minutes from the current meeting.

Phil Wakelyn made some opening comments about the importance of this project to cotton  
growers and that this was a grassroots effort, made up of direct interactions among growers, gins  
and public agencies. The product is manufactured by the Arizona Cotton Research and  
Protection Council (a component of the Arizona Department of Agriculture) and distributed  
directly to growers. He also highlighted the fact that there are no chemical alternatives to  
aflatoxin management and the agricultural industry welcomed an effective biopesticide solution.  
EPA appreciated the innovative approach and direct grower involvement.

Larry Antilla talked about the building of the production facility in Arizona over the last 3 years  
and that about 46,000 acres had been treated. Case studies of aflatoxin reduction figures on  
several farms were reviewed. One farm with approximately 1,000 acres of cotton had previously  
never been able to produce cottonseed below 20 ppb. In 2000, that farm produced seed with  
acceptable aflatoxin contents (<20 ppb) on 82% of its 17 treated fields and in 2001, 86% of  
treated fields produced cottonseed with acceptable aflatoxin contents. Chuck Younger gave a  
personal perspective on the aflatoxin problem in Arizona and noted that growers have committed  
\$2.4 million dollars to the research program. In addition, it was pointed out that the only means  
of mitigation for high aflatoxin levels in seed involves the injection of anhydrous ammonia,  
which is caustic and poses human health risks.

There was a general discussion of the toxicology data (which was unofficially submitted in early  
May). It was generally agreed that the Tween 80 used in the first mammalian study was  
responsible for some effects that were not related to AF36 which was confirmed by the second  
study. The toxicology data constitutes the remaining portion of the registration package to be  
submitted and must be officially submitted before being considered.

- \* Many parts of the current label language were developed due to the lack of toxicology data and  
if justified, can be modified. The combination of acceptable toxicology data, the lack of an  
increase in total *Aspergillus* and a reduction in aflatoxin producing spores all contribute to a  
favorable risk profile due to no change in exposure combined with reduced hazard of AF-36 the  
spores to the environment. Some of the more specific parts to be considered included adding a

\* 2. Refer to the discussion on the label language for perhaps a distinction  
between the two studies

statement that AF36 can be applied to irrigated fields, removal of the buffer statement, wind direction statement, modification of the 12 month storage statement, statement related to inducing vomiting, and perhaps some others. *A label review is required to bring the label in compliance with Agency Section 3 requirements.* There was general discussion about the distribution of the S strain of *A. flavus* with regard to areas that the section 3 registration would include. Initially the registration would cover Arizona and Texas.

In the final topic of discussion Phil Hutton questioned the need for a genetic marker test. He deferred this to John Kough (one of the science reviewers not at the meeting). Peter Cotty reviewed comments and data previously submitted on the reliability of the vegetative compatibility test and the stability of AF36 as a genetic group and its frequent occurrence in the environment and explained the history and reliability of the vegetative compatibility method. He indicated that redundant Vegetative Compatibility Testing (VC testing) was a component of the quality control procedures previously submitted and used in the manufacture of *Aspergillus flavus* AF36. [Personnel are readily trained to perform VC testing and thousands of such analyses are performed annually in order to assess efficacy of AF36 treatments. A DNA based technique could not be practically applied in as robust a manner.] Janet Andersen mentioned that a lot of additional knowledge had been collected since the time the genetic marker test was suggested and that the redundancy of the QA/QC was probably adequate. Janet also suggested that we try to submit most of the information in electronic format in addition to the hard copies to facilitate review.

Now it is up to us to get all of the data in for review so that our goal of section 3 registration by February 2003 can be achieved. While we were previously unaware that this was classified as a public health issue we assume that should add weight to the benefits consideration and prioritize its importance in the review process.

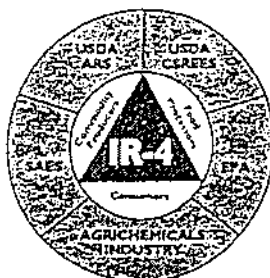
Sincerely,

*Michael Braverman*

Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
FAX (732)932-8481  
braverman@aesop.rutgers.edu

*\*I believe the ? @ hand is more @  
The local level as suppose to exist  
@ Mr Lab. GT agrees - this involves  
esoteric training specifically "tailored"  
for Aspergillus spp.*

cc: Phil Hutton, Shanaz Bacchus, Gail Tomimatsu, Zigfridass Vaituzis, Carl Etsitty, John Kough  
Peter Cotty, Phil Wakelyn, Keith Menchey, Larry Antilla, Chuck Youngker, Bob Holm



**Interregional Research Project No. 4  
Center for Minor Crop Pest Management**

Ms. Shanaz Bacchus and Mr. Phil Hutton  
US EPA  
BPPD/OPP (7511C)  
Crystal Mall No. 2 (Room 902)  
1921 Jefferson Highway  
Arlington, VA 22202

JUL 11 2000

Subject: Pre-registration meeting on Aspergillus  
flavus AF36 on Cotton in Arizona

Dear Ms. Bacchus and Mr. Hutton:

Thank you for arranging the meeting at EPA on June 27, 2000. We found the meeting informative and productive. This letter is to confirm our understanding of the meeting between the US EPA, the Arizona Cotton Research and Protection Council, the National Cotton Council, Arizona Cotton Growers, IR-4 and Dr. Peter Cotty of the USDA/ARS. The following people were present.

US EPA

Michael Watson	OPP/BPPD
Doug Gurian-Sherman	OPP/BPPD
Zig Vaituzis	OPP/BPPD
Phil Hutton	OPP/BPPD
Shanaz Bacchus	OPP/BPPD
John Kough	OPP/BPPD

IR-4

Bill Biehn	Coordinator
------------	-------------

continued .....

Technology Centre of New Jersey  
681 U.S. Highway #1 South • North Brunswick, NJ 08902-3390 • 732/932-9575 • Fax: 732/932-8481

Ms. Shanaz Bacchus and Mr. Phil Hutton (con't)

Arizona Cotton Research and Protection Council

Larry Antilla                      Staff Director

Arizona Cotton Growers Association

Chuck Youngker                  President

USDA/ARS

Peter Cotty                      Research Plant Pathologist

National Cotton Council

Phillip J. Wakelyn              Senior Scientist

The purpose of this meeting was to discuss additional registration requirements needed for a full Section 3 registration for atoxigenic Aspergillus flavus AF 36 for use on cotton in Arizona and the results of Dr. Cotty's research on the airborne spore levels of flavus in treated and untreated fields. Dr. Cotty also presented additional information regarding the widespread occurrence of A. flavus in the natural desert habitats in Arizona as well as other results.

Points Made During Meeting

- Peaks in airborne spore counts of A. flavus occur from September to November. The number of airborne spores of A. flavus in cotton fields treated with A. flavus strain AF36 are not significantly different from the number of airborne spores of A. flavus in untreated fields.
- Acute Pulmonary Toxicity/Pathogenicity Requirement in Mammals.  
This study should be conducted utilizing spores of A. flavus AF36. The dose should be based on 100 times the maximum exposure level possible (i.e.  $450 \text{ cfu/m}^3 \times 100$  equals  $4.5 \times 10^4 \text{ cfu/animal}$ ).
- Avian Acute Pulmonary Toxicity/Pathogenicity Requirement in Bobwhite Quail.  
For AF36, the preferred method of administration is via aerosol inhalation. IR-4 will have EPA review the protocol before initiation of this study.
- Honey Bee Testing Requirement - EPA will provide IR-4 with sample protocols and EPA will review the protocol prepared for AF36 before initiation of this study. A field exposure study would be adequate.
- Avian Acute Oral Toxicity/Pathogenicity Requirement. EPA indicated that this test could be waived. A request for a waiver of this study will be submitted to EPA. Since the avian acute pulmonary toxicity pathogenicity study will be done and since there is an "oral component" to the avian inhalation study, the pulmonary study should be sufficient. Additional scientific rationale will be provided with the formal submission of a waiver request for this study.

continued .....

Page 3

Ms. Shanaz Bacchus and Mr. Phil Hutton (con't)

- An application for an expanded and extended Experimental Use Permit (EUP) involving 80,000 to 95,000 acres as well as a request for an extension of the temporary tolerance exemption will be submitted to EPA in the near future.

Again, we want to thank you for the meeting. Please inform us of any suggested changes or additions to the minutes of this meeting. If we don't hear from you in 30 days, we will assume EPA is in agreement with the above minutes of the meeting.

Sincerely,



William L. Biehn, Ph.D.  
Coordinator  
IR-4 Project

WLB:js

cc: C. Youngker  
L. Antilla  
P. Cotty  
P. Wakelyn  
R. Holm  
J. Baron

rbaccdjydybblbacchusm.wpd

***Conference Call with EPA Thursday, March 13<sup>th</sup>, 2003 at 2:30 PM EST***

The following ten individuals are potential participants in conference call from outside EPA:

Dr. Phil Wakelyn, Senior Scientist, National Cotton Council, Washington, DC

Dr. Michael Braverman, Biopesticide Manager, IR-4, Rutgers University, New Brunswick, NJ

Larry Antilla, Staff Director, Arizona Cotton Research and Protection Council, Phoenix, AZ

Dr. Peter J. Cotty, Research Plant Pathologist, USDA, ARS, SRRC, New Orleans, LA

Dr. Jane F. Robens, National Program Leader, USDA, ARS, Beltsville, MD

Jeff Nunley, Executive Vice President, South Texas Cotton and Grain Association, Victoria, TX

Craig Shook, Chairman of the Board, South Texas Cotton and Grain Association, Victoria, TX

Clyde Sharp, President, Arizona Cotton Growers Association, Phoenix Arizona

Hollis Sullivan, Manager, Valley Cooperative Oil Mill, Harlingen, Texas

## Advances in Utilization of Atoxigenic Strain Technology to Manage Aflatoxin in Commercial Cotton

Larry Antilia, Staff Director, Arizona Cotton Research and Protection Council, Phoenix, AZ ; and Peter J. Cotty, Research Plant Pathologist, USDA-ARS, Southern Regional Research Center, New Orleans, LA

Atoxigenic strains of *Aspergillus flavus* can be highly effective agents for limiting or preventing aflatoxin contamination of Arizona cottonseed. Field tests in Yuma County, conducted from 1996-1998 established that application of atoxigenic strain AF36 on colonized sterile wheat seed was effective at altering the *A. flavus* community associated with the treated crop so that the fungi associated with the crop had reduced potential to produce aflatoxins. This modification was associated with reductions in the aflatoxin content of the crop. The Arizona Cotton Growers Association through the Arizona Cotton Research and Protection Council (ACRPC) initiated development of a facility to manufacture commercially useful quantities of atoxigenic strain material late in 1998. Design and development of the manufacturing process, required equipment, and facility was undertaken by ACRPC in partnership with USDA/ARS. The facility has gone through several development phases and material produced at the facility has been applied to commercial crops since 1999. Collaborative research between ACRPC and USDA/ARS on the use of atoxigenic strain technology to limit aflatoxin contamination of Arizona cottonseed continues with the goal of developing both a theoretical and practical framework by which area-wide reductions in aflatoxin contamination may be achieved. The year 2001 represents the third season of broad scale commercial utilization of atoxigenic strain technology. The atoxigenic strain used is *Aspergillus flavus* AF36. This report addresses progress in the manufacturing, application and evaluation of atoxigenic strain technology on cotton in Arizona during 2001.

During the 2001 crop year a total of eight organized treatment areas representing eleven cotton gins and fifty-two growers were established in Mohave, La Paz, Yuma, Maricopa and Pinal Counties in Arizona. A combined total of 19,975 acres in all areas received AF36 applications with individual areas ranging from 988 to 4492 acres. Analysis of the 2001 crop revealed cumulative effects of large-scale AF36 treatments. *Aspergillus flavus* communities on crop samples from seventy-one (71) randomly selected treated fields averaged 62.9% AF36 and only 1.9% S strain. Statewide individual areas reflected the effects of multi versus first year treatments on seed samples. Roli/Texas Hill (85.2% AF36; 4.1% S), Stanfield (70.4% AF36; 1.3% S), and Paloma (85% AF36; 3.3% S) showed consistently greater AF36 presence on crop than first year treatment sites in Yuma Gila Valley (55.6% AF36 0% S); Parker (42.7% AF36; 7.8% S) and Buckeye (59.6% AF36, 0% S). The observed fungal community changes are particularly significant when considering the extent of AF36 applications over time and space. 2001 treatments were most extensive to date but represent treatment of only 2% of the total available agricultural land mass in the counties affected.

Positive effects on the aflatoxin content of the crop as indicated by commercial analyses were widespread in 2001. In Parker (La Paz County) a large block of cotton (3,200 acres) received its first treatment of AF36. Even though this area was treated over 1 month late, at harvest the gin reported 44% clean seed (below 20 ppb). This represented a significant improvement over the 30-year average of less than 20 percent clean seed. Near Texas Hill (Yuma County) a new treatment area was established in another area with habitually high contamination. Analysis of the harvested crop showed a very high incidence of AF36 and commercial toxin analysis indicated seed aflatoxin content below 10 ppb. In Pinal County, prior to the initiation of AF36 treatments one farm in the Stanfield area had not produced clean seed in 30 years. By the second year of treatments (2000) fourteen of seventeen fields (82%) tested below 20 ppb. In 2001 after a third year of AF36 applications 86% of the fields were below 20 ppb. As a result the grower was able to derive an economic advantage through the sale of clean seed.

Improvements to manufacturing processes and facilities were made in 2002. These improvements resulted in increased product uniformity and improved product quality. Stabilization of incubation procedures were the most significant corrective measure. Incubation conditions will be optimized during 2003. Improvements to facilities for product drying were also designed in 2002 and are currently being fabricated.

Proceedings of the 2002 Aflatoxin Elimination Workshop, San Antonio, Texas, October 22nd-25th, 2002.

Further Comments on the Efficacy of *Aspergillus flavus* AF36 in Response to Questions received March 10, 2003, from USSEPA, OPP, Biopesticides and Pollution Prevention Division

Peter J. Cotty, Ph.D., Research Plant Pathologist, USDA, ARS, SRRC, New Orleans, LA 70124. Phone: 504-286-4391

Michael Braverman, Ph.D., Biopesticide Manager, IR-4, Rutgers University, New Brunswick, NJ 08902. Phone: 732-932-9575

Applications of *Aspergillus flavus* AF36 seek to alter the *A. flavus* communities resident in agricultural fields so that the non-aflatoxin (atoxigenic) strain AF36 is more common and highly toxigenic strains (such as the S strain) are less common. This results in reductions in the average aflatoxin producing potential of *A. flavus* communities associated with treated crops and resident in treated fields.

These are the activities we claim for the product *Aspergillus flavus* AF36. **We do not claim to reduce aflatoxin content to any given level.** In some areas and years, aflatoxin content may exceed 2,000 ppb in the seed and a fairly successful displacement (80%) would only be expected to achieve a reduction to a level in excess of 400 ppb. Yet, in many cases, the industry and particularly the producer living on the farm, would view this as advantageous.

Aflatoxin contamination of cottonseed is monitored in several ways in different areas. In general, it is carefully monitored going into dairy markets. The FDA does not perform this monitoring, although they may do spot checks. Industry performs the analyses. Aflatoxin content of the milk is often monitored carefully and if toxin is detected (at 0.3 ppb) the dairies begin looking for the source (usually corn, cottonseed, or milo). If toxin exceeds 0.5 ppb, the milk must be dumped and the dairy is placed on quarantine. The liability for this generally lies on the provider(s) of the feed.

The FDA has different action levels for cottonseed going into different markets:

- Cottonseed may only contain 20 ppb to be used for dairy cattle.
- Cottonseed containing up to 300 ppb can be fed at beef feedlots (i.e. for finishing cattle).
- Cottonseed meal intended for beef cattle, swine or poultry may contain up to 300 ppb aflatoxin.

Even cottonseed exceeding 300 ppb often has markets. It may be sold to an oil mill where the crush must be carefully monitored to maintain meal below 300 ppb. This seed may be sold to cottonseed brokers that ammoniate the contaminated seed to reduce contamination or it may be sold to markets where vegetable proteins are so highly valued that process methods for dealing with aflatoxin contaminated seeds have been developed (i.e. certain Mexican markets). The aflatoxin content of each lot of seed sold into these markets is generally

known and identified. The quantity of aflatoxins influences the value of the seed. It is more difficult to ammoniate seed that exceeds 2,000 ppb than seed that is only 400 ppb. Seed with lower aflatoxin may be more valuable in secondary markets such as Mexico. Seed with lower aflatoxin is more likely to produce meal with aflatoxin contents acceptable for some uses.

It is not unusual for aflatoxin contents to vary by several orders of magnitude between adjacent fields and across adjacent years. Thus it is not feasible to assess the impact of applications directly on aflatoxin contents. Instead we rely on measurements of successful displacement and on the experience of participating gins and producers. Typically, initial areas to be treated are those that have the severe problems with contamination. An example of this was the first farm we treated in 1996 that had 7,000 ppb the previous year. This selection of fields to participate by producers and gins further complicates the toxin view. Nevertheless, we can and have accurately measured displacement of aflatoxin producers and increases in the incidence of the non-aflatoxin producing AF36 on crops and in soils through both the use of repeated measures tests and analysis of variance in replicated trials. The relationship of this displacement to reductions in contamination has been proven in laboratory, greenhouse, and field-plot tests. In commercial field tests, models using cottonseed oil free fatty acid content as a measure of weathering have also supported this relationship.

See report entitled "Report on Results of Experimental Program on the use of Atoxigenic *Aspergillus flavus* strain AF36 on Cotton Performed Under Experimental Use Permit 69224-EUP-1: Influences Applications on Communities of *A. flavus* Resident in the Soil of Treated Fields and Assessment of Stability of the Atoxigenic Phenotype of *Aspergillus flavus*" (no MRID assigned). Efficacy data can also be found in MRID 43763405 Cotty, P. Hartman, C. (1995) *Aspergillus flavus* Isolate AF36: Product Performance Data.

We are concerned over potential delays in reviewing the newly requested data. We are open to other ways in which to bring this review to a conclusion. While we do not view this as a public health pesticide we can also amend the label to remove the statements pertaining to reductions in aflatoxin and change the label claims only to include displacement of Aflatoxin producing strains of *Aspergillus flavus*. By removing the claim for reducing aflatoxin, AF36 should certainly not be considered a public health pesticide so there is no need to review the efficacy data and the review can be brought to a conclusion. If efficacy is reviewed, it should be based on the reduction of toxigenic strains.

**The above mentioned report provides extensive evidence for the efficacy of *Aspergillus flavus* AF-36 in reducing the proportion of the *A. flavus* community composed of the S strain. The S strain produces very high aflatoxin quantities and is a very significant component of the *A. flavus* community in both Arizona and South Texas. Information on the efficacy of AF-36 in modifying *A. flavus* communities in Texas follows.**

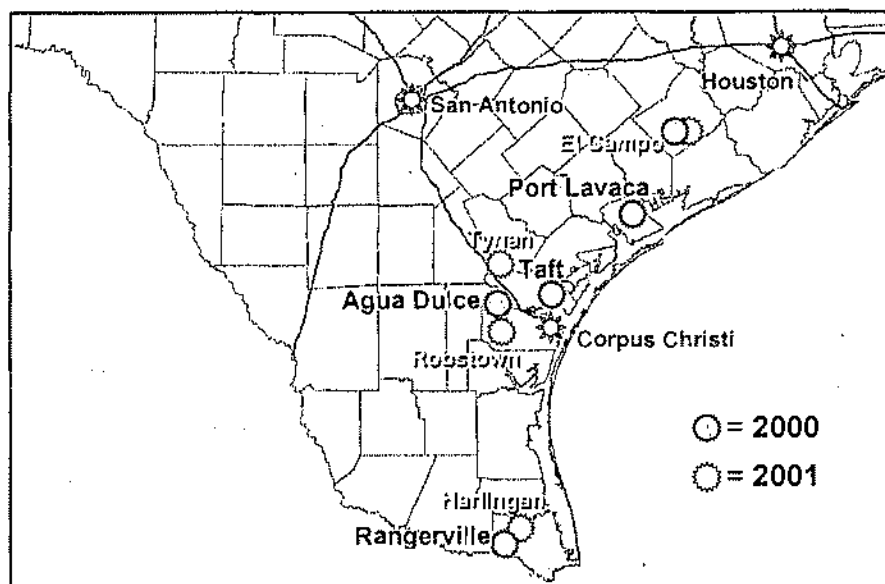
Efficacy of *Aspergillus flavus* AF36 in Texas: Results of Field Tests on the 2000

and 2001 Commercial Cottonseed Crops.

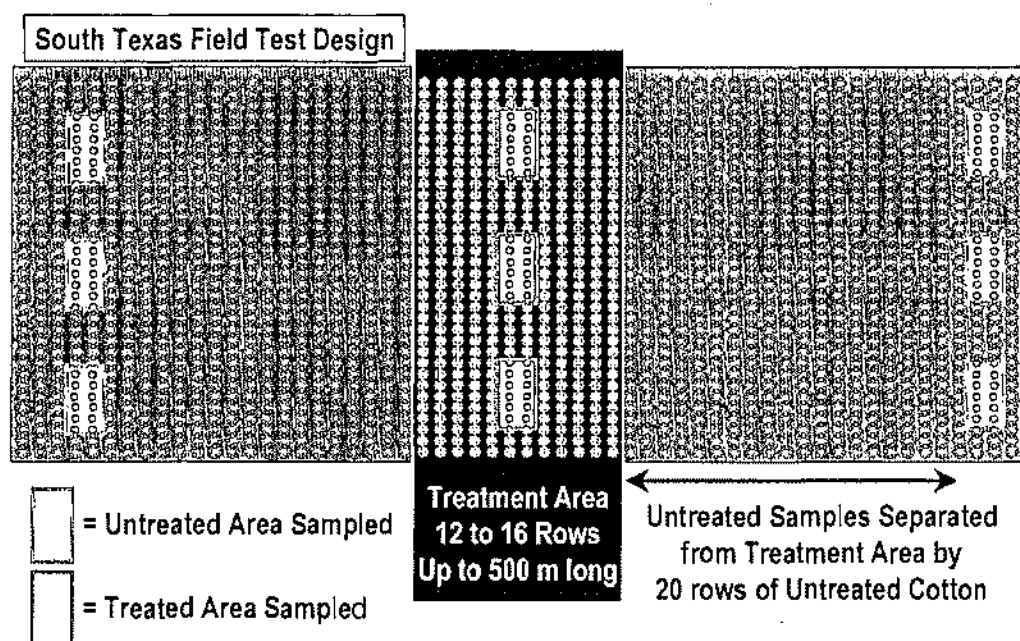
Peter J. Cotty  
Research Plant Pathologist  
USDA-ARS-SRRC  
P.O. Box 19687, New Orleans, LA 70124  
504-286-4391

In order to assess efficacy of soil applied *Aspergillus flavus* AF36 in South Texas, field tests were performed in commercial cotton fields at 9 locations throughout South Texas. Tests extended from Rangerville in the Lower Rio Grande Valley area to El Campo in the Upper Coast area.

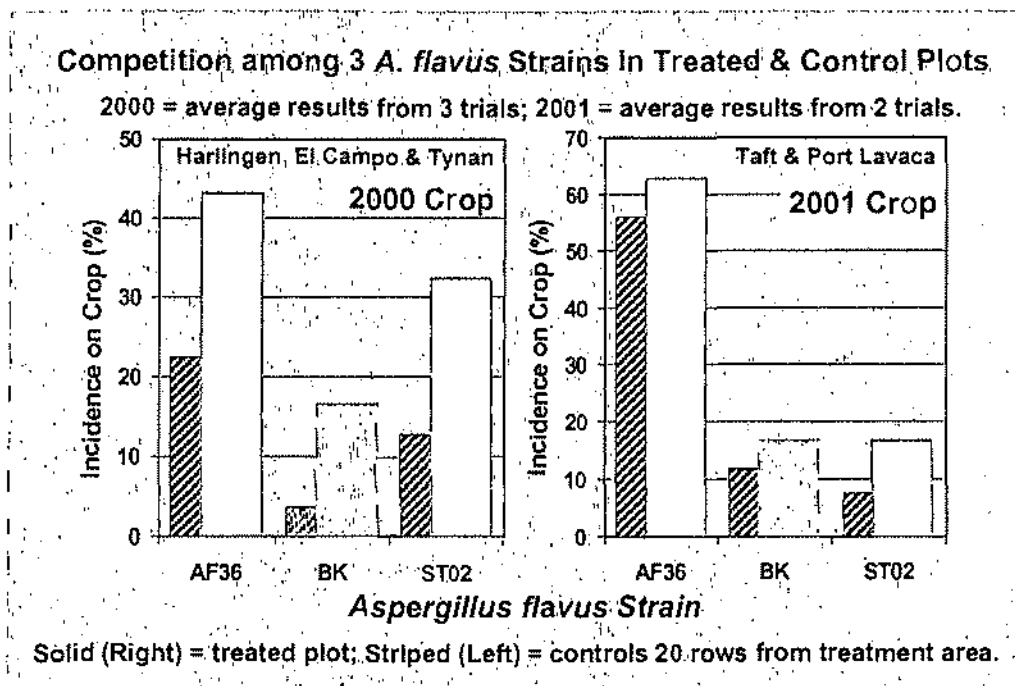
### Locations of South Texas Field Tests in 2000 and 2001



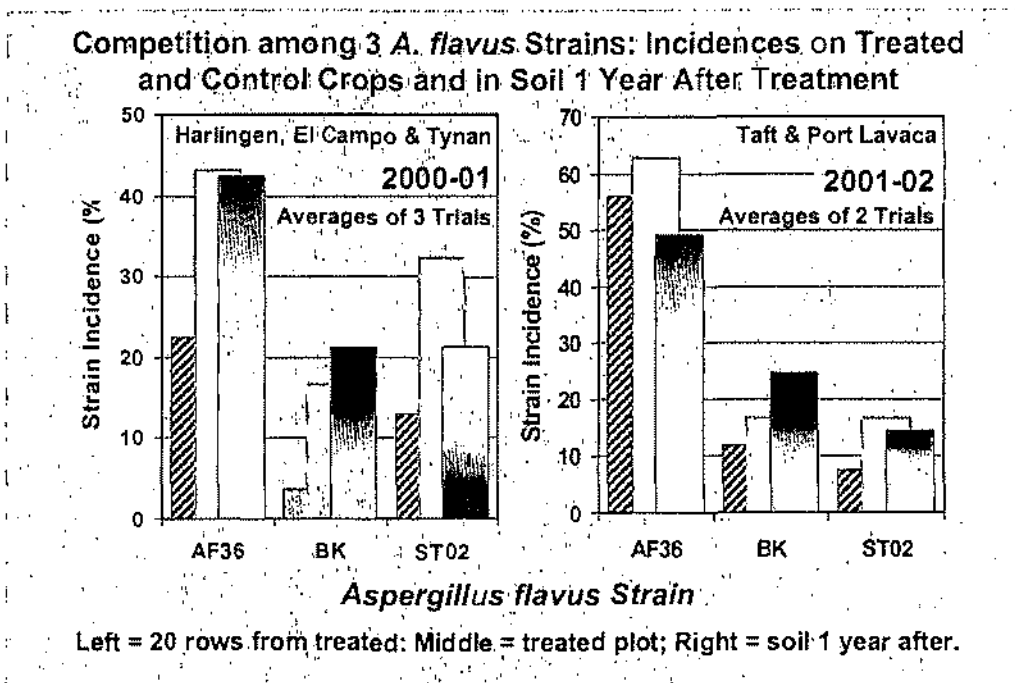
For each test from 0.5 to 1.0 acre of cotton was treated by hand by sprinkling the standard wheat seed formulation of *Aspergillus flavus* AF36 on the soil at the standard rate of 10 lb./acre. In all tests multiple atoxigenic strains of *A. flavus* native to South Texas were evaluated. All strains were applied to the same area at the same rate in order to observe competition among strains and differences among strains in efficacy. Efficacy of atoxigenic strains at displacing the highly toxigenic S strain and other native strains was assessed by characterizing the communities of fungi associated with the mature crop in both treated areas and in untreated control areas separated from the treated areas by 20 rows of untreated cotton (see field test design below).



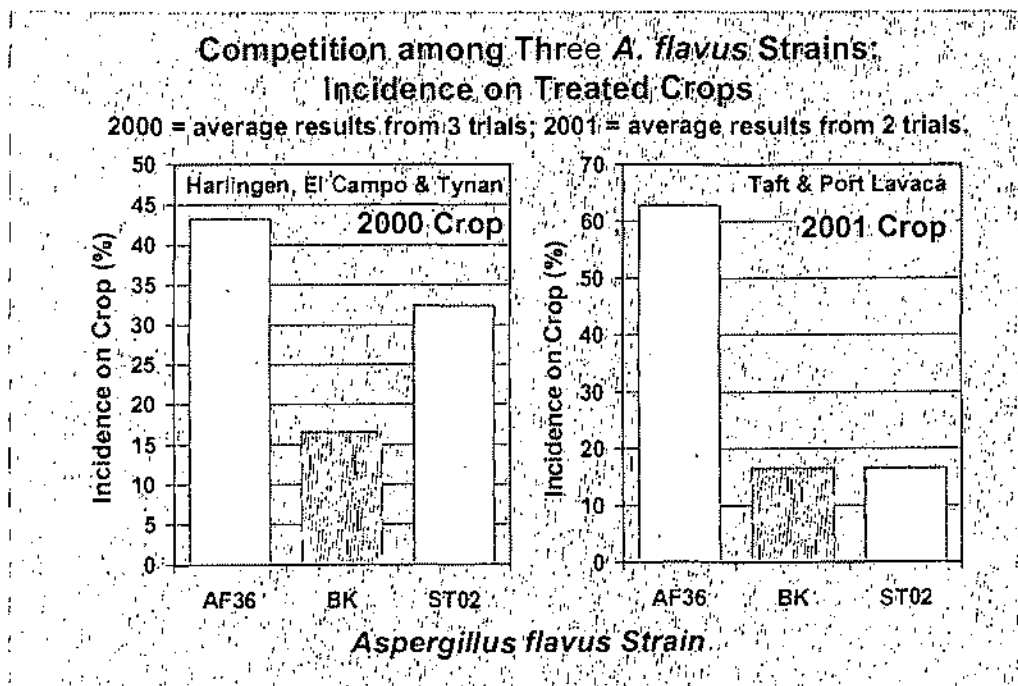
Long-term influences of atoxigenic strains of *A. flavus* have been observed in Arizona. Comparing the communities of fungi resident in the soil just prior to treatment with the community present one year after treatment typically is used to assess this. Such comparisons determine if influences of treatments can be expected to provide a benefit to the environment and crops the second year by reducing the average aflatoxin producing potential of fungi resident in the field across multiple years. The potential for long-term influences of atoxigenic strain applications in South Texas was determined by analyzing the composition of the *A. flavus* communities in the soil of treated plots prior to treatment, with the community structures one year after treatment. Similar comparisons were made contrasting soil in the untreated control plots.



In all tests, AF36 also demonstrated the ability to spread within treated fields across untreated areas. This activity has repeatedly been observed in Arizona and is an aspect of the efficacy of AF36 in displacing aflatoxin producers. The goal of AF36 applications is to modify *A. flavus* communities so that they have a lower potential to produce aflatoxins. The tests in Texas demonstrate great efficacy of AF36 in achieving that goal.



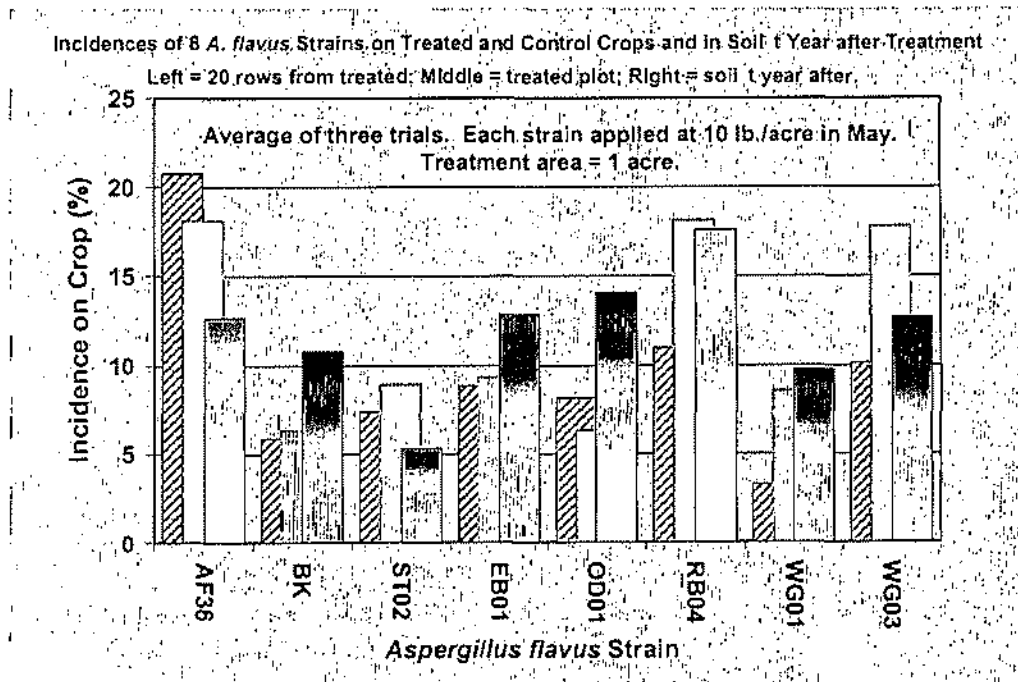
In these studies, three atoxigenic strains (AF36, BK, and ST02) were applied to the treatment area. Incidence of AF36 on treated crops in 2000 and 2001 demonstrate efficacy of *Aspergillus flavus* AF36 at displacing aflatoxin producers during crop colonization.



Both AF36 and the other two-atoxicgenic strains evaluated were effective at spreading from the applied product to the crop and displacing aflatoxin producers during the process. Each strain was applied a single time at 10 pounds per acre. AF36 was the most effective strain in these tests. All strains had efficacy in displacing resident aflatoxin producers.

An important aspect of the efficacy of AF36 in Arizona is the ability of applications to make changes to the composition of *A. flavus* communities in soils that are detectable even the season after application. This allows the possibility of inducing long-term reductions in the aflatoxin-producing potential of *A. flavus* communities resident in fields and thus provides the potential to get additive reductions over time. This allows for long-term reductions in the quantity of aflatoxins in crops and in the environment.

Tests performed in Texas in 2000 and 2001 demonstrated excellent efficacy in producing long-term influences of atoxigenic strain applications similar to those seen in Arizona.



Three tests in 2001 were also performed in which 8 atoxigenic strains were compared for efficacy in ability to competitively exclude aflatoxin producers. *Aspergillus flavus* AF36 demonstrated superior efficacy in these trials as well.

\*Personal privacy information\*



Peter Cotty  
<pjccotty@srcc.ars.usd  
a.gov>

03/12/03 12:59 PM

To: LAntilla@AZcotton.com, PWAKELYN@cotton.org, Shanaz  
Bacchus/DC/USEPA/US@EPA, Phil Hutton/DC/USEPA/US@EPA  
cc: braverman@AESOP.RUTGERS.EDU, Jane Robens  
<JFR@ARS.USDA.GOV>, Jane Robens  
<Jane.Robens@NPS.ARS.USDA.GOV>, jhunley@stcga.org  
Subject: Phone Conference at 2:30 PM EST Thursday March 13th

Dr. Wakelyn asked me to forward the attached list of expected participants in the conference call we will hold tomorrow afternoon.

Dr. Wakelyn will travel to Phil Hutton's office and join the conference call from there.

Please call in at 2:30 PM EST to [REDACTED] The conference code is [REDACTED]

Thank you.

--Peter.

Peter J. Cotty, Ph.D.  
Research Plant Pathologist  
Southern Regional Research Center  
Agricultural Research Service  
United States Department of Agriculture  
1100 Robert E. Lee Blvd.  
New Orleans, LA 70124

pjccotty@srcc.ars.usda.gov  
Phone: 504-286-4391  
FAX: 504-286-4496



participants Doc Call 0305.c

2:30 PM  
AF36  
H-L-g  
1 depends on tables



Gail Tomimatsu  
03/07/03 02:09 PM

To: Phil Hutton/DC/USEPA/US@EPA  
cc: Carl Etsitty/DC/USEPA/US@EPA, Dennis  
Szuhay/DC/USEPA/US@EPA, Janet  
Andersen/DC/USEPA/US@EPA, John  
Kough/DC/USEPA/US@EPA, Shanaz  
Bacchus/DC/USEPA/US@EPA, Zigfridas  
Vaituzis/DC/USEPA/US@EPA  
Subject: Re: Aspergillus flavus AF36..FYI/Data Waivers, TX

Phil, et al.

At this point, I would agree with your guesstimate of 2-4 weeks review time for the 30 page efficacy data. I have not seen efficacy data (i.e., measurement of aflatoxin levels/reductions from harvests of treated vs untreated cotton fields.) for the EUP/registration.

However, I sort of recall John expressing some concern over the "scanty" efficacy data that had been submitted in the past.

I agree with your advice for them to take AZ for now and add big TX later. I think their current EUP "runs" until Dec. 2004, but i'm not sure of the amount of acreage in Texas.

Hope this helps,  
g

Phil Hutton

Phil Hutton  
03/07/2003 01:55 PM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc: Carl Etsitty/DC/USEPA/US@EPA, Dennis  
Szuhay/DC/USEPA/US@EPA, Gail  
Tomimatsu/DC/USEPA/US@EPA, John  
Kough/DC/USEPA/US@EPA, Zigfridas  
Vaituzis/DC/USEPA/US@EPA, Janet Andersen/DC/USEPA/US  
Subject: Re: Aspergillus flavus AF36..FYI/Data Waivers, TX

A late efficacy data submission is going to delay the registration process, I'd say by at least two weeks, possibly 4 (Gail-agree?). We were originally looking to finish this whole thing up by the middle of april (ready for signature, not necessarily signed off). Now I would say more like the end of april at the very best, middle of may more likely. The alternative would be for them to take the AZ only which is supported and seek the addition of TX as an amendment for later. This would save them some time. It would be much faster to do the AZ only and expand the TX EUP to more acres (can do 5000 practically administratively with only Janet's signature), and I bet Jim Jones would quickly sign larger (up to 20K) acres. We need to get this information to the applicant so they understand what is involved as it may mess up their application timing to have to wait the extra time to get the new data reviewed.

Shan- cotton council is calling me daily, but I don't like responding to them until we have informed the official applicant. If you can call IR-4 (representing AZ cotton growers), I can call Phil Wakelyn. Or, maybe better, we can arrange a conference call for all three on Monday (you can call in from flexiplace if we can get the lines).

Phil



Gail Tomimatsu  
03/10/03 08:33 AM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc: Carl Etsitty/OC/USEPA/US@EPA, Dennis  
Szuhay/DC/USEPA/US@EPA, Janet  
Andersen/OC/USEPA/US@EPA, John  
Kough/OC/USEPA/US@EPA, Phil Hutton/OC/USEPA/US@EPA,  
Zigfridas Vaituzis/DC/USEPA/US@EPA  
Subject: Re: Soil/air/ efficacy for AF36 and updates for review; other  
thoughts

Shawn,

Thanks for all your updates and messages on Friday.

In answer to your question: Would you prefer to look at the study MRID 453072-02 or the published paper? I prefer not to look at either study unless necessary. Portions of MRID 453072-02 were acceptable for estimating "background levels" of AF36 (atoxicogenic *Aspergillus*) and total populations of *Aspergillus flavus* (atoxicogenic + toxigenic *Aspergillus flavus*). **However, as I recall, there were no data regarding aflatoxin analyses.** This study was not a guideline study, and was useful to determine appropriate dosage levels of A36 for the avian inhalation study (per memorandum of Tomimatsu and Vaituzis, 2001: "Review of Protocol for Testing the Toxicity/Pathogenicity of the MPCA, *Aspergillus flavus* Strain AF36 (Chemical No.:006456) to Avian Species: DP Barcode:D274694; Case No:03976; Submission:S596777; ID #: 069224-EUP-001)"). The 2001 review is a 3 page memorandum which provides comment on IR-4's proposed avian inhalation study; a DER was not written for this particular review, because of time constraints. I think it is an inefficient use of our time to review this MRID again.

I will be unable to "look at" the **recent** (?) efficacy study until late Tuesday. As Phil and I discussed (via e-mail on Friday), it could take 2 to 4 weeks to review these studies. And, we are uncertain as to whether or not aflatoxin reduction was demonstrated in the data. As many of us are aware, aflatoxin levels are extremely fickle, and are largely dependent on environmental factors more so than population levels of *Aspergillus flavus* (in total).

gail



Gail Tomimatsu

03/10/03 08:33 AM

To: Shanaz Bacchus/DC/USEPA/US@EPA

cc: Carl Etsitty/DC/USEPA/US@EPA, Dennis

Szuhay/DC/USEPA/US@EPA, Janet

Andersen/DC/USEPA/US@EPA, John

Kough/DC/USEPA/US@EPA, Phil Hutton/DC/USEPA/US@EPA,

Zigfridas Vaituzis/DC/USEPA/US@EPA

Subject: Re: Soil/air/ efficacy for AF36 and updates for review; other thoughts

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gail



Gail Tomimatsu  
02/26/03 03:50 PM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc: Zigfridas Vaituzis/DC/USEPA/US@EPA, Joel  
Gagliardi/DC/USEPA/US@EPA  
Subject: A. flavus AF36 on cotton

Shan,

How is the BRAD developing?

A quick scan of the table I provided you several months ago (and below for Joel's benefit), in preparation for your meeting with IR-4 (I was unable to attend) and potential BRAD development indicated a number of ecological test waivers absent (freshwater fish and aquatic invertebrates, estuarine and marine animal testing and possibly an avian oral study, pending review of the avian inhalation study (received from the contractor recently).

Are these waivers forthcoming?

thanks,  
g



EcoReqTable06-2002.wp

Shanaz Bacchus



Shanaz Bacchus  
03/07/03 11:05 AM

To: Gail Tomimatsu/DC/USEPA/US@EPA, Zigfridas  
Vaituzis/DC/USEPA/US@EPA, John Kough/DC/USEPA/US@EPA,  
Carl Etsitty/DC/USEPA/US@EPA  
cc: Dennis Szuhay/DC/USEPA/US@EPA, Phil  
Hutton/DC/USEPA/US@EPA  
Subject: Re: Aspergillus flavus AF36..FYI/Data Waivers, TX

Discussion with Mike Braverman, as Phil suggested:

1. Apparently Peter Cotty does have efficacy data for TX. By mid next week, he will be sending (by FEDEX) the 30 page paper which is in the publication stages. Peter has to write up a brief summary and the forms have to be signed by Larry Antilla. They are trying their best not to use an EUP in TX this year, and figure that the TX efficacy data will be acceptable to us.
2. Gail, in view of this development, can you do the avian inhalation review first, then the data waiver (DW) reviews? Do you want Joel to help with the extra reading of the paper and write-up for the DW review? I looked at the DW submission... looks like the same rationale was repeated for all the requests, then some nuances for each specific guideline.
3. Carl, John, a formal form capturing the data waiver requests, which have already been granted for the health effects for the EUP, will also be submitted next week.

Please remember, that most of the DW requests have already been reviewed and that you only need to look at the new material which is coming in. I am just tying up loose ends for the records by getting them to include these formal requests as Phil, Zig asked me to do.

I had given you packages of the DERs which have been previously used for the issuance of the EUP. If you need any other information, do let me know. I will be working at home on Monday, but can access my email and voicemail.

Thanks  
shawn  
Phone: 703-308-8097



Gail Tomimatsu

03/07/03 12:30 PM

To: Shanaz Bacchus/DC/USEPA/US@EPA

cc:

Subject: Re: papers for docket....AF36

yes, thank you.



Gail Tomimatsu  
03/07/03 12:11 PM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc: Zigfridas Vaituzis/DC/USEPA/US@EPA, John  
Kough/DC/USEPA/US@EPA, Phil Hutton/DC/USEPA/US@EPA,  
Carl Etsitty/DC/USEPA/US@EPA, Dennis  
Szuhay/DC/USEPA/US@EPA, Joel  
Gagliardi/DC/USEPA/US@EPA, Alan  
Reynolds/DC/USEPA/US@EPA  
Subject: papers for docket....AF36

Shawn,  
Possible response needed, please: Phil, John and/or Zig  
FYI: Dennis, Carl, Alan and Joel

With respect to the stack of papers (i assume they were sent by the Cotton Council, and are available in the public literature) that you wanted to give me on Wednesday, i had another thought/question, post-meeting. Perhaps Phil, John, and Zig can provide some guidance on how to handle these items; something like a section for unreviewed submissions, or public comments (i seem to recall that we've handled similar submissions for the Bt Crops in this manner). Some of the submission might have relevance to each of the hazard chapters.

I would like to take a quick look (at a minimum) at these papers, after all. At our meeting on Wednesday, I wanted to focus only on the materials that needed secondary review immediately before writing the hazard assessments. If you've given the papers to Joel, that's okay with me.

Maybe I can take a quick look sometime next week--perhaps Tuesday or Thursday (?).

Thanks everyone for your advice and help.

gail

p.s.: Shawn in response to your questions sent today: 2 can you do the avian inhalation review first, then the data waiver (DW) reviews? i hope to finish the peer reviews next week.

Do you want Joel to help with the extra reading of the paper --what paper? and write-up for the DW review? Unless Zig V. and Phil thinks that this idea will save time, I feel that the plans we discussed on Wednesday should stand. The "Eco data" table I sent you a few days ago should help clarify some of your questions. Joel is certainly welcome to look over the waiver rationale sent in electronically, however. By the way, I understood that these will not have MRIDs. How are we to refer to these waiver rationales in the risk assessment chapters and BRAD?



Gail Tomimatsu  
03/05/03 01:44 PM

To: Shanaz Bacchus/DC/USEPA/US@EPA, Alan  
Reynolds/DC/USEPA/US@EPA  
cc: Joel Gagliardi/DC/USEPA/US@EPA, Zigfridas  
Vaituzis/DC/USEPA/US@EPA, Dennis  
Szuhay/DC/USEPA/US@EPA, Phil Hutton/DC/USEPA/US@EPA,  
Carl Etsitty/DC/USEPA/US@EPA  
Subject: Aspergillus flavus AF36..FYI and thank you..eco-risk assessment...

Shawn and Phil,

Thanks for bringing everyone up to speed this morning, and providing an "idea" of next steps.

To make sure we are on the same page (more or less), please note and comment on the following "action items" with respect to the secondary review of the 3 eco-studies, several test waiver rationales and the ecological risk assessment.

**MRID # 45798102** (Avian inhalation test): secondary review to be performed by GST  
**MRID # 45739103** Supplemental Information for Endangered Avian species (Plover): secondary review to be performed by GST  
**MRID # 45739102** Field Testing of Pollinators and Honey Bee Testing: secondary review to be performed by Alan Reynolds (since Robyn is on AL); he believes he can get to it the first of next week.

Data Waivers: GST will do the primary reviews, Joel G. can "peer review" (for his training)

I hope to have the ecorisk assessment (more or less complete) by March 28. Zig, would you want some time before then to peer review?

Comments/Corrections?  
Alan and Zig--thanks for your help!  
Thanks,  
g



Gail Tomimatsu

02/27/03 10:08 PM

To: Shanaz Bacchus/DC/USEPA/US@EPA

cc:

Subject: Re: rough draft/AF36

Thanks, Shan!

we'll "plow" through it next week. =D

This should be helpful in our discussion on Wednesday.

g



Gail Tomimatsu

02/27/03 08:31 AM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc: Phil Hutton/DC/USEPA/US@EPA, Dennis  
Szuhay/DC/USEPA/US@EPA, Zigfridas  
Vaituzis/DC/USEPA/US@EPA, Joel  
Gagliardi/DC/USEPA/US@EPA  
Subject: A. flavus AF36

Shan,

Just a heads up on the meeting next week, which I had extended an option to attend. Please try to attend, so that we can brief Dennis, Zig and I on development of the risk assessment and BRAD. Joel would like to sit in on the meeting, but he will not peer review the 3 studies.

Please let me know if the time is inconvenient for you, and whether you believe 45 minutes will be sufficient for the briefing. I reserved the small conference room from 9:15 to 10 am

Thanks,  
gail



Gail Tomimatsu  
02/26/03 03:50 PM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc: Zigfridas Vaituzis/DC/USEPA/US@EPA, Joel  
Gagliardi/DC/USEPA/US@EPA  
Subject: A. flavus AF36 on cotton

Shan,

How is the BRAD developing?

A quick scan of the table I provided you several months ago (and below for Joel's benefit), in preparation for your meeting with IR-4 (I was unable to attend) and potential BRAD development indicated a number of ecological test waivers absent (freshwater fish and aquatic invertebrates, estuarine and marine animal testing and possibly an avian oral study, pending review of the avian inhalation study (received from the contractor recently).

Are these waivers forthcoming?

thanks,  
g



EcoReqTable06-2002.wp



Gail Tomimatsu

02/25/03 03:05 PM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc: Phil Hutton/DC/USEPA/US@EPA  
Subject: Re: Good news/more AF36 [E]

Shawn,

we have another chinese fire drill that takes precedence now.



Gail Tomimatsu

02/25/03 03:04 PM

To: Shanaz Bacchus/DC/USEPA/US@EPA

cc:

Subject: Re: Good news/more AF36



Gail Tomimatsu  
02/13/03 04:57 PM

To: Shanaz Bacchus/DC/USEPA/US@EPA  
cc: Carl Etsitty/DC/USEPA/US@EPA, John  
Kough/DC/USEPA/US@EPA, Phil Hutton/DC/USEPA/US@EPA,  
Zigfridas Vaituzis/DC/USEPA/US@EPA  
Subject: Re: Good news/more AF36

Shawn,

Thank you for the reviews which I found on my chair, not on my shelf as you indicated in your message.

Approximate timeline: 2-4 weeks; as i have 2 other microbial pesticides that I am currently working on, in addition to the BtCry3Bb BRAD, response to comments.

Have they applied for an extended EUP?

g



Gail Tomimatsu

01/29/03 03:18 PM

To: Carol Frazer/DC/USEPA/US@EPA

cc: OPP BPPD

Subject: Re: Endangered Species?

Carol,

Please add Plant Incorporated Protectants : in the paragraph from Zig's earlier comments regarding endangered species assessments and evaluation of exposure potential to intended applications of MPCAs **AND** the Plant Incorporated Protectants (EUPs and registrations):

"microbial pesticides (Pips risk characterization and assessment follow that of current guidelines for microbial pesticides) are mostly species specific, we also take a look at the habitat of the endangered species that are phylogenically related to the target pest to see if there is an overlap of the breeding and feeding habitat with the crop that the MPCA and Plant Incorporated Protectants will be used on, or planted in. Most of the crops that our a.i.'s are used on do not overlap with endangered species habitats (have not overlapped to date) so we can make a no-effect finding on the basis of no exposure. "



Gail Tomimatsu

01/22/03 10:19 AM

To: William Schneider/DC/USEPA/US@EPA, Russell Jones/DC/USEPA/US@EPA  
cc: Alan Reynolds/DC/USEPA/US@EPA, Anne Ball/DC/USEPA/US@EPA, Barbara Mandula/DC/USEPA/US@EPA, Chris Wozniak/DC/USEPA/US@EPA, John Kough/DC/USEPA/US@EPA, Linda Hollis/DC/USEPA/US@EPA, Mike Mendelsohn/DC/USEPA/US@EPA, Phil Hutton/DC/USEPA/US@EPA, Robyn Rose/DC/USEPA/US@EPA, Shanaz Bacchus/DC/USEPA/US@EPA, Sharlene Matten/DC/USEPA/US@EPA, Susanne Cerrelli/DC/USEPA/US@EPA, Suzanne Krolkowski/DC/USEPA/US@EPA, Zigfridas Vaituzis/DC/USEPA/US@EPA

Subject: Re: Only the best! - or - microbial mush!

Require efficacy data on poop soup?

Efficacy data as Barbara points out is not a FIFRA requirement (at the time of submission), unless the pesticidal products make public health claims. Getting efficacy data for these "pesticides", e.g., poop soup, bugs-in-a-jug, snake oils, is very difficult-- even though one might believe it would be in the best interests (from a marketing/liability perspective) for a manufacturer to keep track of his/her products and their claims.

We can say that the "efficacy data" is necessary for us to establish use patterns; whereby we might be able to do a (less than scientific) risk assessment. There is a mechanism in FIFRA which I think might allow us to request efficacy data, but the manufacturer has to justify their case that it is the public's benefit (sic, "in the public's interest") to use their "pesticide". It would also be incumbent upon them to make the case that the use(s) of their product will not cause adverse risks to human health and the environment. This is a "public-interest" finding; and could use a considerable amount of resources (time and FTEs), which this Division is seriously in lack of.

I agree with Zig: Let those potential registrants go through the "painstaking" regulatory process just like the other producers. I predict we will have to do a lot of "waving" (purposely misspelled).

If we exempt these products from FIFRA, we might be opening ourselves up to generic labelling, such as: a buyer beware clause ("caveat emptor"; user/consumer alert) that this product, while "registered with/regulated by" the EPA has not been fully evaluated for human health or environmental risks. Add to that, some statement that the product may not provide the expected pest control for all situations. I think that such statements could undermine our present and future capabilities as a "lead" Agency with Federal authority (sic oversight) to protect human health and the environment by "permitting" pollution of intended use sites with microbial mush which is probably present.

I also will be unable to attend your meeting.

Calendar Entry  
Meeting

Subject: Peer Reviews and EcoRisk Eval. A. flavus AF36 Location:  
Begins: Wed 03/05/2003 09:15 AM Entry type: Meeting  
Ends: Wed 03/05/2003 10:00 AM  
Chair: Gail Tomimatsu/DC/USEPA/US

Invitations already sent

To: Dennis Szuhay/DC/USEPA/US@EPA, Joel Gagliardi/DC/USEPA/US@EPA, Zigfridas Vaituzis/DC/USEPA/US@EPA  
cc: Phil Hutton/DC/USEPA/US@EPA; Shanae Bacchus/DC/USEPA/US@EPA

☐ Pencil Ink  
☐ Mark Private  
☐ Notify me  
Categorize:

Description:  
Please let me know if this meeting is inconvenient.

Purpose: Brief meeting to discuss ecological risk assessment and BRAD development for a Sec. 3 registration for atoxigenic *Aspergillus flavus* AF36 to displace toxigenic *A. flavus* in cotton fields; and to "deliver" 3 submitted studies which require peer review. The 3 studies were reviewed by one of the contractors (Oak Ridge) and include the following Tier 1 test studies: Avian Inhalation Test (based on EPA-approved protocol) and a Field Test of Pollinators and Honey Bee tests for partial fulfillment of OPPTS 850.3040 (Ecological Effects), and OPPTS Guideline 885.4380 (Microbial Test Guidelines). The 3rd submission contains "Supplemental Information on Potential Toxicity to the Migratory Bird - Plover spp."

3/28 - Review 5 ↔ 4/4 - OGC - signature 4/21 Keith Matthews [Lavel Caliste, Michelle Kiser]  
Exposure differs to Carl - Mammalian 1 Roby - hbee  
Cotton pollen [Alan R. R. - hbee - 2° 2 DW - Gail 1°  
Eco DW → Gail - DW - avian. } 3 Zig 2°  
Joel - Hutton } Peer review } HRIDS Mann. DW  
DW

Tech around EUP.TX making it easy - Extension.  
Check FR Notice

Further Comments on the Efficacy of *Aspergillus flavus* AF36 in Response to Questions received March 10, 2003, from USSEPA, OPP, Biopesticides and Pollution Prevention Division

Peter J. Cotty, Ph.D., Research Plant Pathologist, USDA, ARS, SRRC, New Orleans, LA 70124. Phone: 504-286-4391

Michael Braverman, Ph.D., Biopesticide Manager, IR-4, Rutgers University, New Brunswick, NJ 08902. Phone: 732-932-9575

Applications of *Aspergillus flavus* AF36 seek to alter the *A. flavus* communities resident in agricultural fields so that the non-aflatoxin (atoxicogenic) strain AF36 is more common and highly toxigenic strains (such as the S strain) are less common. This results in reductions in the average aflatoxin producing potential of *A. flavus* communities associated with treated crops and resident in treated fields.

These are the activities we claim for the product *Aspergillus flavus* AF36. **We do not claim to reduce aflatoxin content to any given level.** In some areas and years, aflatoxin content may exceed 2,000 ppb in the seed and a fairly successful displacement (80%) would only be expected to achieve a reduction to a level in excess of 400 ppb. Yet, in many cases, the industry and particularly the producer living on the farm, would view this as advantageous.

Aflatoxin contamination of cottonseed is monitored in several ways in different areas. In general, it is carefully monitored going into dairy markets. The FDA does not perform this monitoring, although they may do spot checks. Industry performs the analyses. Aflatoxin content of the milk is often monitored carefully and if toxin is detected (at 0.3 ppb) the dairies begin looking for the source (usually corn, cottonseed, or milo). If toxin exceeds 0.5 ppb, the milk must be dumped and the dairy is placed on quarantine. The liability for this generally lies on the provider(s) of the feed.

The FDA has different action levels for cottonseed going into different markets:

- Cottonseed may only contain 20 ppb to be used for dairy cattle.
- Cottonseed containing up to 300 ppb can be fed at beef feedlots (i.e. for finishing cattle).
- Cottonseed meal intended for beef cattle, swine or poultry may contain up to 300 ppb aflatoxin.

Even cottonseed exceeding 300 ppb often has markets. It may be sold to an oil mill where the crush must be carefully monitored to maintain meal below 300 ppb. This seed may be sold to cottonseed brokers that ammoniate the contaminated seed to reduce contamination or it may be sold to markets where vegetable proteins are so highly valued that process methods for dealing with aflatoxin contaminated seeds have been developed (i.e. certain Mexican markets). The aflatoxin content of each lot of seed sold into these markets is generally

known and identified. The quantity of aflatoxins influences the value of the seed. It is more difficult to ammoniate seed that exceeds 2,000 ppb than seed that is only 400 ppb. Seed with lower aflatoxin may be more valuable in secondary markets such as Mexico. Seed with lower aflatoxin is more likely to produce meal with aflatoxin contents acceptable for some uses.

It is not unusual for aflatoxin contents to vary by several orders of magnitude between adjacent fields and across adjacent years. Thus it is not feasible to assess the impact of applications directly on aflatoxin contents. Instead we rely on measurements of successful displacement and on the experience of participating gins and producers. Typically, initial areas to be treated are those that have the severe problems with contamination. An example of this was the first farm we treated in 1996 that had 7,000 ppb the previous year. This selection of fields to participate by producers and gins further complicates the toxin view. Nevertheless, we can and have accurately measured displacement of aflatoxin producers and increases in the incidence of the non-aflatoxin producing AF36 on crops and in soils through both the use of repeated measures tests and analysis of variance in replicated trials. The relationship of this displacement to reductions in contamination has been proven in laboratory, greenhouse, and field-plot tests. In commercial field tests, models using cottonseed oil free fatty acid content as a measure of weathering have also supported this relationship.

See report entitled "Report on Results of Experimental Program on the use of Atoxigenic *Aspergillus flavus* strain AF36 on Cotton Performed Under Experimental Use Permit 69224-EUP-1: Influences Applications on Communities of *A. flavus* Resident in the Soil of Treated Fields and Assessment of Stability of the Atoxigenic Phenotype of *Aspergillus flavus*" (no MRID assigned). Efficacy data can also be found in MRID 43763405 Cotty, P. Hartman, C. (1995) *Aspergillus flavus* Isolate AF36: Product Performance Data.

We are concerned over potential delays in reviewing the newly requested data. We are open to other ways in which to bring this review to a conclusion. While we do not view this as a public health pesticide we can also amend the label to remove the statements pertaining to reductions in aflatoxin and change the label claims only to include displacement of Aflatoxin producing strains of *Aspergillus flavus*. By removing the claim for reducing aflatoxin, AF36 should certainly not be considered a public health pesticide so there is no need to review the efficacy data and the review can be brought to a conclusion. If efficacy is reviewed, it should be based on the reduction of toxigenic strains.

The above mentioned report provides extensive evidence for the efficacy of *Aspergillus flavus* AF-36 in reducing the proportion of the *A. flavus* community composed of the S strain. The S strain produces very high aflatoxin quantities and is a very significant component of the *A. flavus* community in both Arizona and South Texas. Information on the efficacy of AF-36 in modifying *A. flavus* communities in Texas follows.

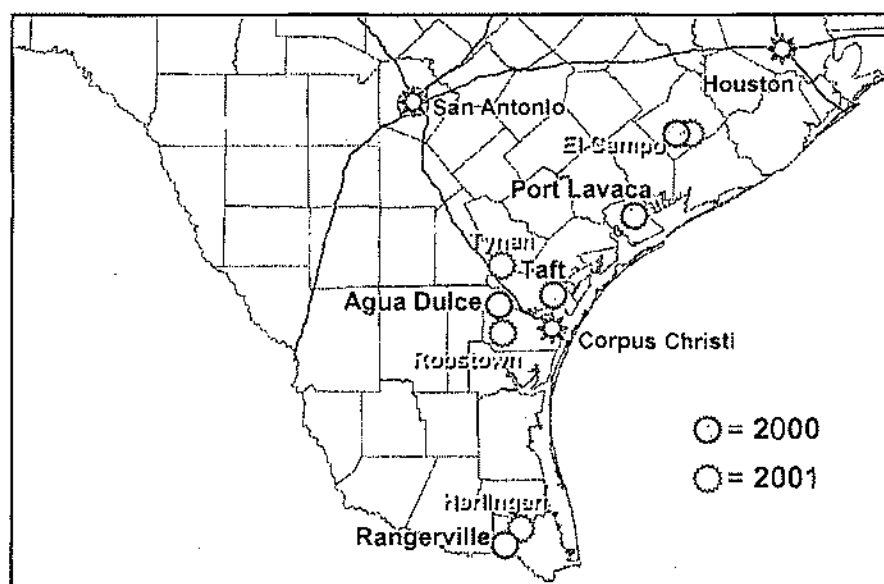
Efficacy of *Aspergillus flavus* AF36 in Texas: Results of Field Tests on the 2000

and 2001 Commercial Cottonseed Crops.

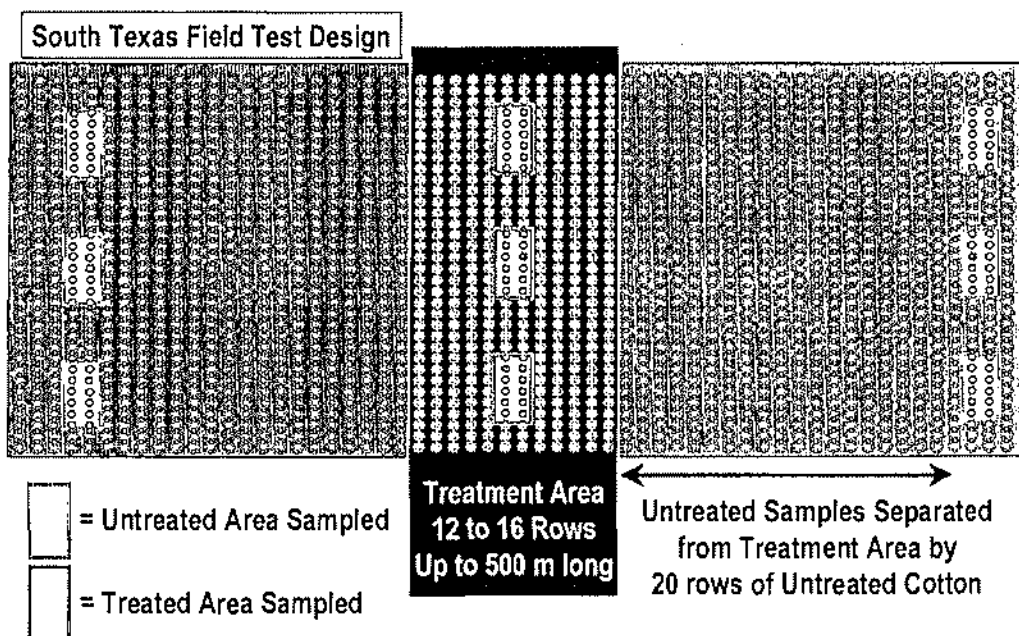
Peter J. Cotty  
Research Plant Pathologist  
USDA-ARS-SRRC  
P.O. Box 19687, New Orleans, LA 70124  
504-286-4391

In order to assess efficacy of soil applied *Aspergillus flavus* AF36 in South Texas, field tests were performed in commercial cotton fields at 9 locations throughout South Texas. Tests extended from Rangerville in the Lower Rio Grande Valley area to El Campo in the Upper Coast area.

#### Locations of South Texas Field Tests in 2000 and 2001

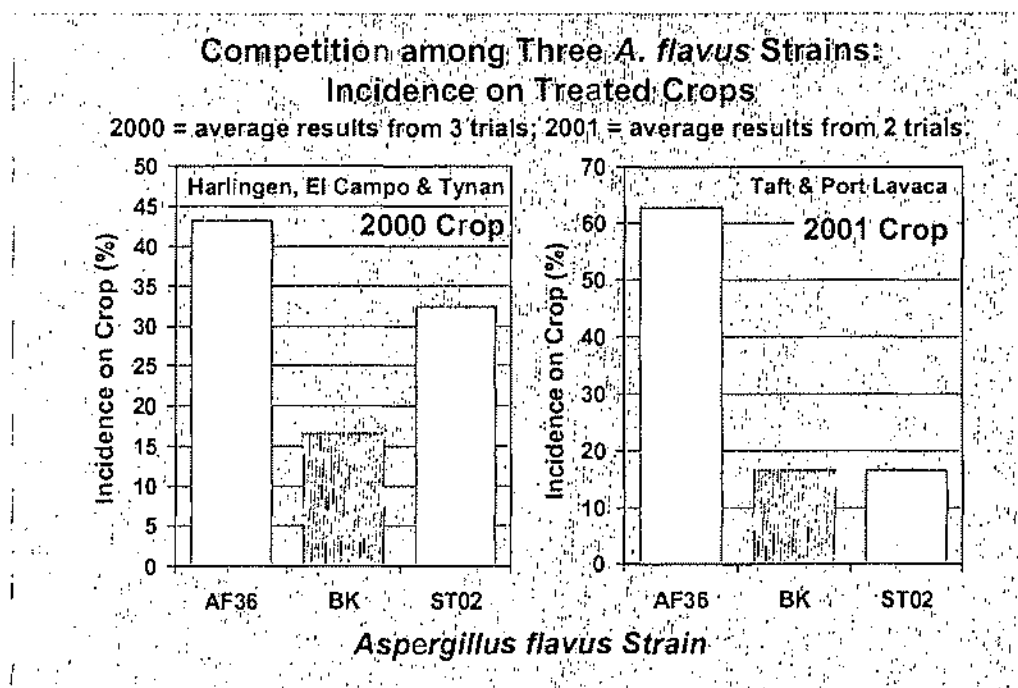


For each test from 0.5 to 1.0 acre of cotton was treated by hand by sprinkling the standard wheat seed formulation of *Aspergillus flavus* AF36 on the soil at the standard rate of 10 lb./acre. In all tests multiple atoxigenic strains of *A. flavus* native to South Texas were evaluated. All strains were applied to the same area at the same rate in order to observe competition among strains and differences among strains in efficacy. Efficacy of atoxigenic strains at displacing the highly toxigenic S strain and other native strains was assessed by characterizing the communities of fungi associated with the mature crop in both treated areas and in untreated control areas separated from the treated areas by 20 rows of untreated cotton (see field test design below).



Long-term influences of atoxigenic strains of *A. flavus* have been observed in Arizona. Comparing the communities of fungi resident in the soil just prior to treatment with the community present one year after treatment typically is used to assess this. Such comparisons determine if influences of treatments can be expected to provide a benefit to the environment and crops the second year by reducing the average aflatoxin producing potential of fungi resident in the field across multiple years. The potential for long-term influences of atoxigenic strain applications in South Texas was determined by analyzing the composition of the *A. flavus* communities in the soil of treated plots prior to treatment, with the community structures one year after treatment. Similar comparisons were made contrasting soil in the untreated control plots.

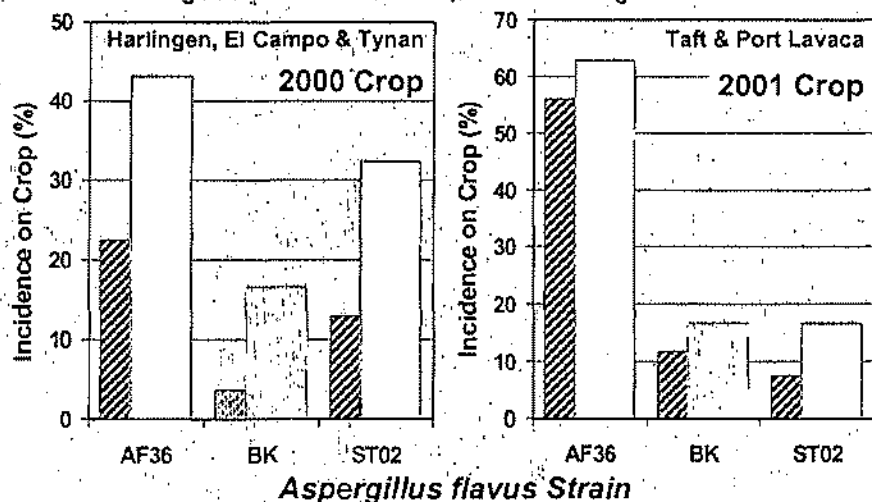
In these studies, three atoxigenic strains (AF36, BK, and ST02) were applied to the treatment area. Incidence of AF36 on treated crops in 2000 and 2001 demonstrate efficacy of *Aspergillus flavus* AF36 at displacing aflatoxin producers during crop colonization.



Both AF36 and the other two atoxigenic strains evaluated were effective at spreading from the applied product to the crop and displacing aflatoxin producers during the process. Each strain was applied a single time at 10 pounds per acre. AF36 was the most effective strain in these tests. All strains had efficacy in displacing resident aflatoxin producers.

### Competition among 3 *A. flavus* Strains in Treated & Control Plots

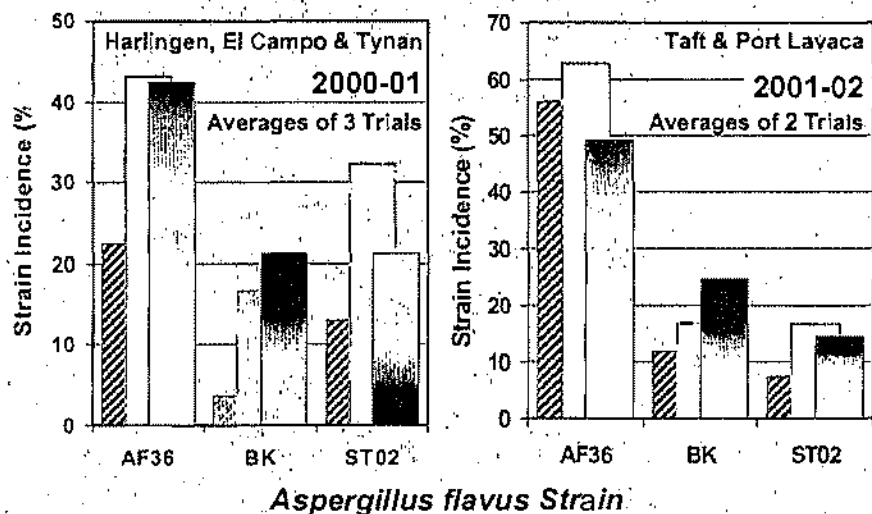
2000 = average results from 3 trials; 2001 = average results from 2 trials.



Solid (Right) = treated plot; Striped (Left) = controls 20 rows from treatment area.

In all tests, AF36 also demonstrated the ability to spread within treated fields across untreated areas. This activity has repeatedly been observed in Arizona and is an aspect of the efficacy of AF36 in displacing aflatoxin producers. The goal of AF36 applications is to modify *A. flavus* communities so that they have a lower potential to produce aflatoxins. The tests in Texas demonstrate great efficacy of AF36 in achieving that goal.

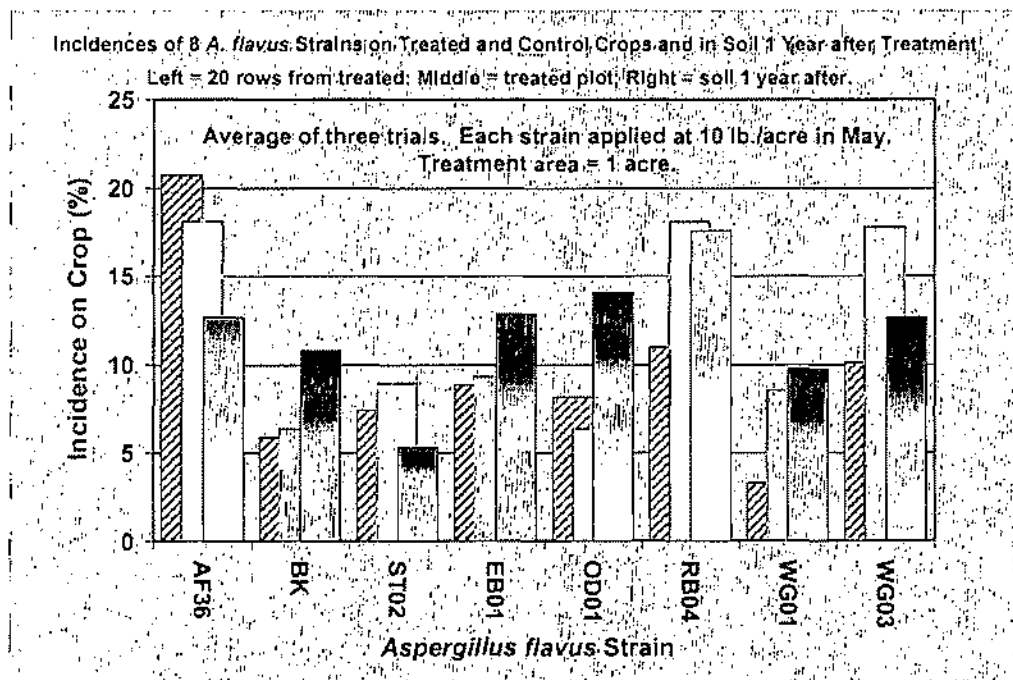
### Competition among 3 *A. flavus* Strains: Incidences on Treated and Control Crops and in Soil 1 Year After Treatment



Left = 20 rows from treated; Middle = treated plot; Right = soil 1 year after.

An important aspect of the efficacy of AF36 in Arizona is the ability of applications to make changes to the composition of *A. flavus* communities in soils that are detectable even the season after application. This allows the possibility of inducing long-term reductions in the aflatoxin-producing potential of *A. flavus* communities resident in fields and thus provides the potential to get additive reductions over time. This allows for long-term reductions in the quantity of aflatoxins in crops and in the environment.

Tests performed in Texas in 2000 and 2001 demonstrated excellent efficacy in producing long-term influences of atoxigenic strain applications similar to those seen in Arizona.



Three tests in 2001 were also performed in which 8 atoxigenic strains were compared for efficacy in ability to competitively exclude aflatoxin producers. *Aspergillus flavus* AF36 demonstrated superior efficacy in these trials as well.



UNITED STATES ENVIRONMENTAL PROTECTION AGENCY  
WASHINGTON, D.C. 20460

SEP 16 1998

OFFICE OF  
PREVENTION, PESTICIDES AND  
TOXIC SUBSTANCES

Dr. Bill Biehn, Coordinator  
Interregional Research Project No.4  
681 US Highway #1 South  
North New Brunswick, NJ 08902

Dear Dr. Biehn:

**Subject: Pre-registration meeting on *Aspergillus flavus* AF36  
on Cotton in Arizona**

The Agency has reviewed your submission dated May 7, 1998, regarding the minutes of the meeting with certain members of the Biopesticides and Pollution Prevention Division (BPPD). The points in your submission were numbered 1 through 11 to facilitate communication on certain points. The team members have provided the following comments on the meeting minutes:

Item 3 - "The purpose of the EUP.....need to be evaluated."

Your claim in this project is to eliminate the toxigenic strain of *A. flavus*. It is highly unlikely that you will eliminate aflatoxin. Accurate claims should address reduction of the toxigenic strain or of aflatoxin, and if possible, which of the aflatoxins you plan to reduce.

Item 7 - "Concern was expressed.....Section 3 registration."

A blue folder was presented with anecdotal information to support a request to waive the requirement for the avian toxicity studies. Requests to waive data must be supported by sound scientific information. In your data waiver request, please clarify:

- (a) what preferred habitats for birds are available within the treatment area;
- (b) the proximity of such habitats to the cotton growing areas;
- © what the exposure of birds is likely to be to *A. flavus* AF36 during pesticide application and during the growing season.

Item 8 - "It was also pointed out.....avian toxicology studies."

Clarify whether wheat fields are adjacent to and/or in close proximity to cotton fields in Arizona. Include either a study plan or what procedure you plan to use to monitor the exposure and effects on birds in the treated area.

Item 9 - "Since *A. flavus* occurs.....Experimental Use Permit."

(a) The potential pathogenicity of the active ingredient must be addressed. A label warning of potential pathogenicity and a requirement for appropriate Personal Protective Equipment will be required on labels issued for use of *A. flavus* AF36.

(b) Requests to waive data requirements for environmental fate may be supported by the submission of information regarding the microbial ecology of both aflatoxin-producing and non-aflatoxin producing *A. flavus* in Arizona.

Item 11 - "EPA indicated that a description of the manufacturing.....We agreed to provide it." With reference to the folder entitled "Recommendations for setting up a large-scale AF-36 wheat production facility in Phoenix, Arizona", dated April 1997, the team was of the opinion that the extrapolation from the scale-up from 2 cubic feet to 100 cubic feet may be problematic and that further quality control measures may be required.

It is good that you mention that verification will be requested relative to temperature maintenance within the sterilization procedure. During the scale-up, the time required for cooling from 37°C to 31°C after inoculum addition may increase. It is feasible that without adequate Quality Assurance controls, the potential for contamination by extraneous microorganisms can occur. Since the systems appear to have agitators, it may be possible to cool the hot sterile seeds with HEPA filtered air. If this is not practical and you have alternative proposals, describe what Quality Assurance and Quality Control measures you plan to take to minimize contamination during the manufacturing process.

Also of concern are:

- (a) There was some question about whether the manufacturer was aware of the sterile techniques required for solid state fermentation, (see memo dated April 6, 1998, from Joe Ploski (USDA) to Tom Chirkot (Patterson-Kelley Co.). Please verify that steps will be taken to implement those sterile techniques and to train manufacturing staff to maintain those techniques.
- (b) Is there an alternative non-porous material to pillow cases for use in the drying oven? Can steps be taken to contain the potential dissemination of spores from the pillow cases during the transport of the AF36-treated wheat seeds to the drying oven?
- © Include in your description of the manufacturing process, the steps taken to monitor air quality to ascertain product integrity.
- (d) The wheat seeds treated with *A. flavus* AF36 must be differentiated by way of color from other wheat seeds used for planting or processing. Include the amounts of the dye/coloring material used in your Confidential Statement of Formula and a description of the method to color the seeds in your manufacturing process.

If you would like to discuss these matters any further, do not hesitate to call Shanaz Bacchus at 703-308-8097.

Sincerely,

Phil O. Hutton, Chief  
Microbial and Plant Pesticides Branch  
Biopesticides and Pollution  
Prevention Division

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

SEP 12 1998

Dr. Bill Biehn, Coordinator  
Interregional Research Project No.4  
681 US Highway #1 South  
North New Brunswick, NJ 08902

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CONCURRENCES							
SYMBOL	7511C	7511C					
SURNAME	BACCHUS	HATTO					
DATE	9/10/98	9/10/98					

(b) Requests to waive data requirements for environmental fate <sup>review</sup> must be supported by the submission of information regarding the microbial ecology of both aflatoxin-producing and non-aflatoxin producing *A. flavus* in Arizona.

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Sincerely,



Phil O. Hutton, Chief  
Microbial and Plant Pesticides Branch  
Biopesticides and Pollution  
Prevention Division

A. flavens AF 36  
 Mtg CST - Conf RMA  
 GROUP

4/15/98

NAME	GROUP	PHONE
Michael Watson	BPPD	703-308-9118
DOUG GURION-SNERMAN	BPPD	703-308-8117
Gail Tomimatsu	BPPD	703-308-8543
Chris Wozniak	BPPD	703-605-0513
PAT CIMINO	RD/Minor Crops 518's	703-308-9357
Ed Minch	AZ Dept of Agric.	(602) 542-0954
Phil Hutton	BPPD	703-308-8260
John Maguire	Cotton Council	202-745-7805
Shauaz Bachus	BPPD	703-308-8077
Bill Biehn	IR-4	732-932-9575
Gene [unclear]	USDA/ARS	301 504-5581
Jack Norton	IR-4	(732) 932-9575
Chuck [unclear]	AZ Cotton Growers	602-919-6615
LARRY [unclear]	AZ Cotton Council	602 438-0055
Clyde Sharp	AZ Cotton Growers	520-785-9317
* Phil Wakelyn	National Cotton Council	FAX 202 483 4040 202-745-7805
Peter [unclear]	USDA/ARS	504-256-7391
JOHN KOUGH	EPA   BPPD	703-308-8267

OPTIONAL FORM 99 (7-90)

FAX TRANSMITTAL

# of pages 1

To Phil Wakelyn	From Shauaz Bachus
Dept/Agency Nat'l Cotton Council	Phone # 703-308-8097
Fax # 202-483-4040	Fax # 703-308-7026

NSN 7540-01-317-7368 5099-101 GENERAL SERVICES ADMINISTRATION

OPTIONAL FORM 99 (7-90)

FAX TRANSMITTAL

# of pages 1

To Bill Biehn	From Shauaz Bachus
Dept/Agency IR-4	Phone # 703-308-8097
Fax # 732-932-8481	Fax # 703-308-7026

NSN 7540-01-317-7368 5099-101 GENERAL SERVICES ADMINISTRATION



Date:

4-21-98

Number of pages including cover sheet:

**FAX**

To:

Ms. Shanaz Bacchus

Fax number:

CC:

From:

P. Wakelam

Phone:

202-745-7805

Fax number:

202-483-4040

**REMARKS:**

☐ Urgent

☐ For your review

☐ For your info

☐ Please comment

Thank you for FAXing the attendance list.

Attached is the letter sent to you last year following the review of Dr. Cotty's research. Dr. Cotty indicated that they had not received any reply to this letter.

Need answer to <sup>the</sup> letter —

May 28, 1997

Ms. Shanaz Bacchus  
U.S. Environmental Protection Agency  
Biopesticides and Pollution Prevention Division  
Mailcode (7501W)  
401 M St. SW  
Washington, DC 20460

As sent  
*Christina*

Dear Ms. Bacchus:

This letter is to confirm our understanding of the meeting between US EPA, IR-4, USDA/ARS and National Cotton Council at your offices on April 10, 1997. The following people were present:

USEPA

Phil Hutton	OPP/BPPD
Shanaz Bacchus	OPP/BPPD
Doug Gurian-Sherman	OPP/BPPD
John Kough	OPP/BPPD
Cindy Schaffer	OPP/BPPD
Gail Tomimatsu	OPP/BPPD

USDA/ARS

Peter Cotty	Research Plant Pathologist
Jane Robens	National Program Leader, Food Safety and Health

IR-4

Christina Hartman	Biopesticide manager
-------------------	----------------------

National Cotton Council

Phil Wakelyn	Senior Scientist, Environmental Health & Safety
--------------	---

The purpose of this meeting was to determine if the efficacy data currently being generated by Dr. Cotty under an EUP would be sufficient to support a section 3 registration for *Aspergillus flavus* AF36. Strain AF 36 is used to displace aflatoxin producing strains in the cotton crop. Because of the human health implications, efficacy data is required for a full registration and it is necessary that this data be satisfactory to the Agency.

Presented materials

Dr. Cotty opened his presentation with an overview of aflatoxin contamination of cottonseed. Points covered included significance to industry, commercial sampling and analysis for aflatoxins, national versus state regulations, practices and policies, national distribution, and high variability. He next described the two phases of aflatoxin contamination and the

predisposing factors and the current technologies for prevention of contamination of cottonseed.

Dr. Cotty then described the complex communities of *Aspergillus flavus* vegetative compatibility groups. He included a discussion on vegetative compatibility analysis as it pertains to field test analysis. Existing efficacy data and mechanisms of action were reviewed.

Greenhouse experiments have shown that *Aspergillus flavus* interferes with aflatoxin contamination when introduced into damaged cottonbolls at equal or 1/2 the conidial concentration of the aflatoxin producing strain. Presence of AF 36 in bolls or cultures is always associated with reductions in aflatoxin concentrations. Field plot experiments have shown that AF 36 displaces aflatoxin producing strains under cotton producing conditions in Yuma, Arizona and that the end result is a fungal community associated with the crop that has a lower potential to produce aflatoxins. This community change is associated with reductions in aflatoxin contamination. Field plot studies have shown that the aflatoxin content of the crop is directly and inversely related to the incidence of *Aspergillus flavus* AF36. Increases in the incidence of *Aspergillus flavus* AF36 in the greenhouse and field plots consistently result in decreases in aflatoxin concentration in cottonseed. From this it is clear that when the percent of the *Aspergillus flavus* community composed of AF36 is increased, crop vulnerability to aflatoxin contamination is decreased and the quantity of aflatoxin in the crop is reduced compared to what would have been present if AF 36 was not present.

Next Dr. Cotty showed the design for commercial field tests. A chart was shown that showed the distribution of the wheat seed (inoculum) after commercial application. A map of the distribution of the aflatoxin contamination among fields surrounding the treated field at Mohawk Valley test area 1 was shown to illustrate 1996 results. A chart with the influence of gin data on aflatoxin content of the commercial crop in 1995 and 1996 and a bar chart comparing all seed lots from the Mohawk gin with the treated field.

A map of incidence of AF36 in Mohawk Valley test area 1 in soil prior to application and a map of incidence of AF36 on the crop after ginning were used to show the spread of the isolate. Tables presented included aflatoxin levels in Mohawk Valley test area 1, AF36 incidence in Mohawk Valley test area 1, aflatoxin levels in Mohawk Valley test area 2, AF36 incidence in Mohawk Valley test area 2, aflatoxin levels in Yuma Valley test area and AF36 incidence in Yuma Valley test area.

Dr. Cotty next explained the relationship between the concentration of aflatoxin contamination in BGYF seed and the percent Free Fatty Acid content. As free fatty acid content increased, the concentration of aflatoxin in the crop increased. The relationship for untreated fields differed markedly from the relationship for treated fields. Aflatoxin content increased much faster with free fatty acid content in untreated fields. An overhead was shown with aflatoxin content of BGYF seed measured, predicted, average of surrounding fields, and range in surrounding fields.

The total aflatoxin content of cottonseed in untreated fields was also correlated ( $R^2 = 0.8223$ ) with free fatty acid content. Free fatty acids form when cottonseed is exposed to high humidity. Thus, the correlations indicate that as the seed was exposed to high humidity, aflatoxin increased and the increases were greater when the exposure was greater. This agrees with the general picture seen for cottonseed from this area in 1996 where the quantity of aflatoxin increased with harvest date. The latter harvested cotton would be the cotton exposed to humidity to the greatest extent. The total aflatoxin content of cottonseed from treated fields

was also significantly correlated ( $R^2 = 0.9942$ ) with free fatty acid content. However, with the combined data (from treated and untreated fields) the correlation was poor ( $R^2 = 0.2659$ ). This is because the relationship between free fatty acid content and aflatoxin was very different in treated and untreated fields. Aflatoxin content increased with free fatty acid content at a much slower rate in treated fields than in untreated fields. However, a good correlation ( $R^2 = 0.8661$ ) exists between free fatty acid content and the number resulting from multiplying aflatoxin content times the percent of the *A. flavus* community composed of AF36. This supports the contention that the difference between treated and untreated fields in the relationship between aflatoxin content and free fatty acid content is attributable to increased incidence of AF36 in treated fields. The correlations indicate that AF36 reduced the aflatoxin content of seed from treated fields. A very strong correlation ( $R^2 = 0.978$ ) exists with the combined data set between free fatty acid content and the amount of aflatoxin predicted if AF36 was not present. This strong relationship indicates that the presence of AF36 in the commercial fields is a major factor influencing aflatoxin content. The predicated aflatoxin content was calculated by assuming that AF36 caused a proportional linear reduction in aflatoxin content. This assumed all toxin came from isolates other than AF36 and the influence of AF36 resulted in simple reductions in contamination through competitive exclusion. Thus an incidence of AF36 of 50% would cause a 50% reduction in aflatoxin and cottonseed from that field would have a predicted level four times of that measured, etc.).

In conclusion, Dr. Cotty went over the experimental plans for 1997. These include: A) sampling of soil within and nearby fields treated in 1996 in order to assess long-term influences of AF36 application; B) sampling of soil from new fields to be treated in 1997; C) treatment of approximately 500 acres of cotton with AF36 in the Yuma and Mohawk Valleys; D) analysis of ginned cottonseed from the treated fields for AF36, aflatoxins, and free fatty acid content.

#### Discussion

In general comments, Phil Hutton stated that Dr. Cotty was on the right track. John Kough pointed out that the following three areas of data were important to the Agency for the section 3 scientific evaluation. The first area is levels of aflatoxin in cottonseed from treated and non-treated areas. The second is data showing displacement of aflatoxin producing strains by AF36. The third is the data on correlation of free fatty acid with aflatoxin. A list of studies and examples of data generated in 1996 are attached to confirm that these studies are acceptable to EPA for the section 3 registration.

As stated by the Agency, data collected should reflect the goal of less aflatoxin in the treated fields. The data should associate displacement of strains with aflatoxin reduction. Phil Hutton suggested that the overall picture and trends would be given consideration since it can be expected that AF36 can move into adjacent fields thus making some control fields comparable to the treated.

We look forward to receiving your confirmation of the above understanding.

Sincerely,

*Christina L. Hartman*

Christina L. Hartman, Ph.D.  
Biopesticide Manager  
Office of IR-4

## Studies Performed in 1996 to Assess Efficacy of *Aspergillus flavus* AF36

### I. Comparison of Aflatoxin levels in treated and nearby fields.

Seed cotton was harvested commercially. Gins were asked to segregate seed from individual treated and nearby fields on a field by field basis and to keep the seed separate until sampling was accomplished. A single sampling from each field was taken according to the Arizona commercial feed law and this was analyzed for aflatoxin content by an independent laboratory. Six replicated seed samples from each field were taken for analyses at the Southern Regional Research Laboratory. In order to separate toxin forming during the first and second phases of contamination, fluorescent seed was separated from non-fluorescent seed. Aflatoxin contents of the two seed categories were determined separately for each of the six replicate samples for each field.

#### Examples of Data Generated:

Aflatoxin concentrations in BGYF seed both from fields treated with *Aspergillus flavus* AF36 in 1996 and from nearby untreated fields

Treated field	Aflatoxin B <sub>1</sub> in BGYF seed (ppb)			
	From treated fields		From nearby fields	
	Measured <sup>a</sup>	Predicted <sup>b</sup>	Actual average	Actual range
Barkley 44	3	4,732	4,065	73 to 8,056
Stuhr 3	1,648	62,516	10,004	7,113 to 13,515
Sharp 204E	1,509	18,207	14,794	3,194 to 33,055

<sup>a</sup> BGYF seed was sorted from non-fluorescent seed and analyzed for aflatoxin content separately. Values are averages of 6 replicates. Each replicate is the average of two analyses.

<sup>b</sup> Predicted aflatoxin values were calculated from the treated field's free fatty acid content by using the formula of the least square regression line for the correlation between percent free fatty acid and aflatoxin concentration for seed from untreated fields.

#### Aflatoxin Content of Cottonseed Harvested in Yuma Valley Test Area

Field	Type	Aflatoxin (ppb)			Overall
		Commercial	BGYF	Non-BGYF	
44	Treated	0	3	8	8
95	Adjacent	8	73	1	1
29	Adjacent	ND	8,056	44	51
43	Adjacent	ND	ND-Pima	15	15

ND = not determined.

### Aflatoxin Content of Cottonseed Harvested in Mohawk Valley Test Area I

Field	Type	Aflatoxin (ppb)			Overall
		Commercial	BGYF	Non-BGYF	
204 E	Treated	19	1,509	129	138
204 W	Adjacent	180	3,194	53	65
205E	Adjacent	341	33,055	845	855
205W	Adjacent	341	13,984	751	987
201	Adjacent	92	9,934	389	398

### Aflatoxin Content of Cottonseed Harvested in Mohawk Valley Test Area II

Field	Type	Aflatoxin (ppb)			Overall
		Commercial	BGYF	Non-BGYF	
3	Treated	600	1,648	464	471
4	Adjacent	200	9,385	99	164
12	Near	ND	7,113	3	10
23	Near	ND	13,515	69	83

ND = not determined.

## II. Influence of applications on composition of *A. flavus* communities.

Soils within treated and nearby fields were sampled and the incidence of *Aspergillus flavus* AF36 in the *Aspergillus flavus* community resident in the fields was determined by vegetative compatibility analysis. The *Aspergillus flavus* community resident on the crop after harvest was also sampled and the incidence of AF36 determined. The incidences of AF36 in the soil and on the crop were compared.

### Examples of Data Generated:

#### Influence of Applications on Incidence of AF36 in Yuma Valley Test Area

Field	Type	Incidence of <i>Aspergillus flavus</i> AF36 (%)	
		In Soil Prior (# Isolates)	On Cottonseed (# Isolates)
44	Treated	3.0% (157)	74.6% (60)
95	Adjacent	2.1% (131)	7.9% (65)
29	Adjacent	3% (68)	31.2% (55)
43	Adjacent	ND	34.0% (55)

ND = not determined.

Influence of Applications on Incidence of AF36 in Mohawk Valley Test Area I

Field	Type	Incidence of <i>Aspergillus flavus</i> AF36 (%)	
		In Soil Prior (# Isolates)	On Cottonseed (# Isolates)
204 E	Treated	1.1% (154)	91.6% (69)
204 W	Adjacent	4.0% (72)	61.7% (57)
205E	Adjacent	ND	56.9% (72)
205W	Adjacent	0% (58)	27.1% (70)
201	Adjacent	2.6% (67)	42.5% (60)

ND = not determined.

Influence of Applications on Incidence of AF36 in Mohawk Valley Test Area II

Field	Type	Incidence of <i>Aspergillus flavus</i> AF36 (%)	
		In Soil Prior (# Isolates)	On Cottonseed (# Isolates)
3	Treated	8.6% (151)	98.6% (71)
4	Adjacent	0% (70)	23.5% (60)
12	Near	ND	26.4% (72)
23	Near	6.4% (65)	11.2% (71)

ND = not determined.

**III. Long-term and area-wide influences of applications on composition of *A. flavus* communities.**

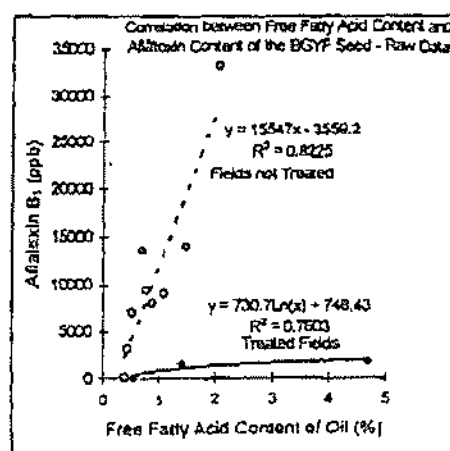
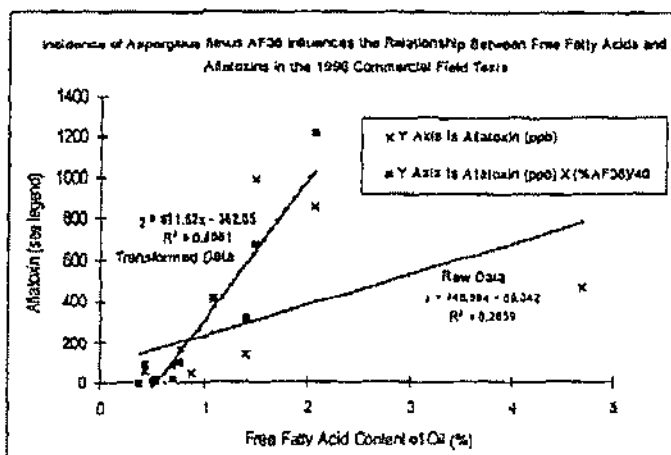
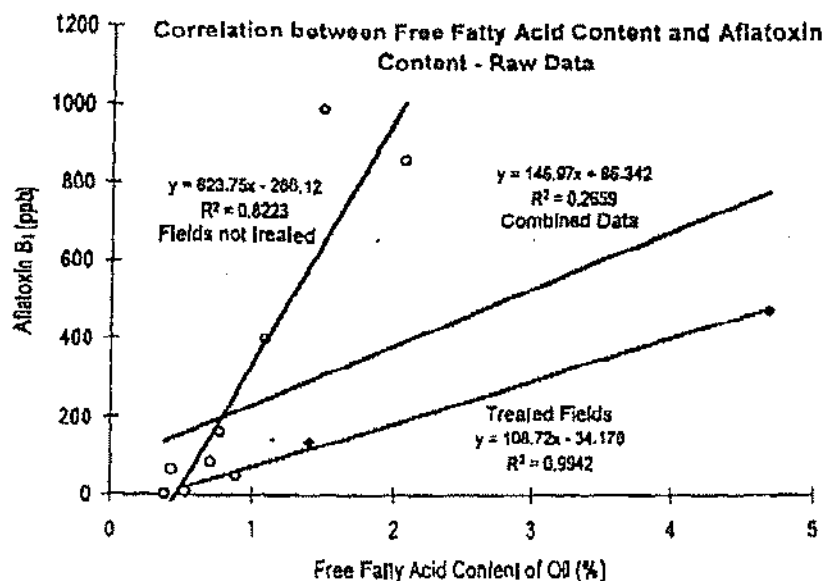
During 1997 the incidence of AF36 in soils withing fields sampled in 1996 will be determined in order to assess long-term and area-wide influences of applications. The quantity of fields will be expanded to included fields treated for the first time in 1997. Data will not be available for these comparisons until spring 1998.

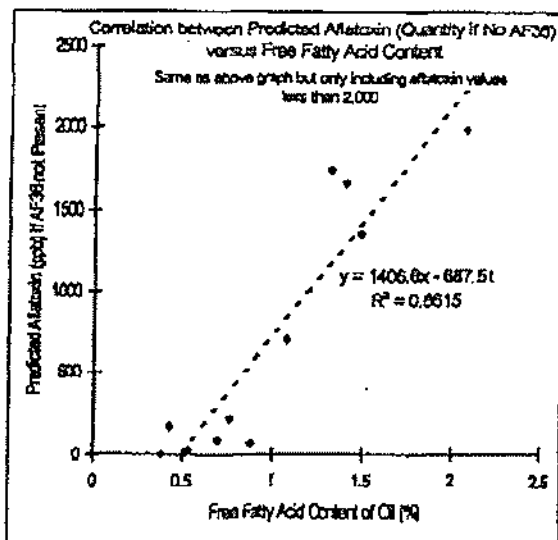
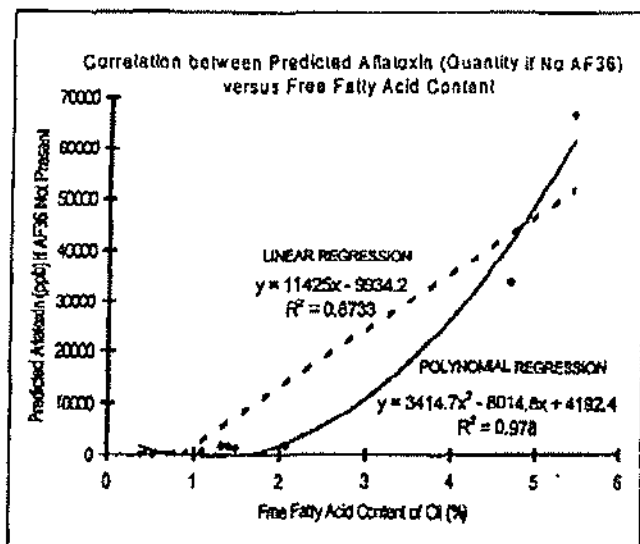
**IV. Model based analysis of influences of applications on aflatoxin contamination using Free Fatty Acid Content of Oil to indicate the extent of crop weathering.**

A subsample of each replicate seed sample (six per field) from each field was sent to a commercial laboratory (Mid-Continent Laboratories, Inc., Memphis, TN) for Free Fatty Acid Analysis. Analyses were performed by official methods.

Correlations were tested between aflatoxin concentrations and free fatty acid content for treated, untreated, and combined data.

### Examples of Data Generated:





### Predicted Aflatoxin Levels if *Aspergillus flavus* AF36 was Absent

#### 1996 Commercial Field Tests

Field	Free Fatty Acids (%)	Measured Aflatoxin (ppb)	Aflatoxin if no AF36 (ppb)
Yuma 29	0.88	51.00	74.20
Yuma 95	0.38	0.68	0.74
Mk1-205W	1.48	987.00	1,354.76
Mk1-204W	0.43	65.00	169.79
Mk1-205E	2.07	855.00	1,985.79
Mk1-201	1.09	398.45	693.57
Mk2-4	0.77	164.45	215.00
Mk2-12	0.52	9.91	13.46
Mk2-23	0.70	83.05	93.56
Yuma1-44	0.53	8.00	31.61
Mk1-204E	1.40	138.00	1,655.87
unknown	1.32	1,284.36	1,735.86
Mk2-3	4.68	470.82	33,896.04
unknown	5.43	667.75	66,775.00

The following corrects a transcriptional error in the submitted version of the letter:

A very strong correlation ( $R^2=0.978$ ) exists with the combined data set between free fatty acid content and the amount of aflatoxin predicted if AF36 was not present. This strong relationship indicates that the presence of AF36 in the commercial fields is a major factor influencing aflatoxin content. The predicted aflatoxin content was calculated by assuming that AF36 caused a proportional linear reduction in aflatoxin content. This assumed all toxin came from isolates other than AF36 and the influence of AF36 resulted in simple reductions in contamination through competitive exclusion. Thus an incidence of AF36 of 50% would cause a 50% reduction in aflatoxin and cottonseed from that field would have a predicted level twice that of the measured aflatoxin content (a field with 75% AF36 would have a predicted level four times of that measured, etc.).

*Aspergillus flavus* AF36 (Atoxigenic strain): Deficiencies and Status of studies from DERs

DER C. Schaffer 2/20/96

DER C. Schaffer 4/23/96

Deficiencies: Health Effects

1. QA/QC

QA/QC addressed

- A. Vegetative compatibility analysis
- A. Microbial contamination
- A. Quantity of conidia by turbidity analysis

2. Sensitizer

A. Not sensitizer in Cotty's lab

3. Taxonomic description of strain  
need colony morphology,  
photomicrographs

A. Submitted.

4. Amt of moisture to be limited

A. Store dry on label. do not expose to  
relative humidity >80% prior to use.

5. Either use respirator or provide  
pulmonary study (No toxicity/pathog.  
pulmonary study submitted for EUP).

A. Used respirator.

6. Need Acute Oral

A. Acute oral done LD50 > 50g/kg.  
Check Tox/path and for how long?

DER G. Tomimatsu 4/24/96

Deficiencies: Ecological Effects

Need, sent letter for timeline  
4/28/97

1. Non-target Mammal, avian honey bee,  
observed stonebrood in honeybee

Recommended avian tox/path  
(N. bobwhite) incl. post mortem and  
histopath.

2. Wildlife toxicity?

Protocols for these??

3. Non-target plants Waived  
Ubiquitous

3. Reported from lit. Aspergillosis in pigs, sheep  
cattle, horses, horses, dogs, birds, insects,  
housefly, termites

4. Endangered spp. not expected in cotton fields.

Differentiate spp. in soil  
[Efficacy - up from  $\neq$  reg. ones]

AVIAN  
Honeybee  
Fish  
Daphnia

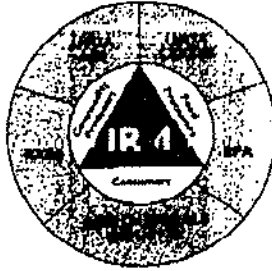
sec 15 — more to be added

- ① Meredith J. } (1) Write Pat Cimini (nicer use)  
② Pat Cimini } (2) " Meredith Johnson.

A Kamel Celeste

Republic ? Feb 11.

B re: A = licence to sell  
maybe 5000 A. without J. signature



Interregional Research Project No. 4  
Minor Crop Pest Management

April 2, 1998

TO: Phil Hutton  
Shanaz Bacchus

FROM: Bill Biehn *Bill Biehn*

SUBJECT: EPA Preregistration Meeting on Aspergillus flavus  
AF36 on Cotton in Arizona.  
Wednesday April 15, 1998 at 1:00 p.m.

Attached is the Agenda and a list of the participants representing USDA/ARS, the cotton growers and the Arizona Department of Agriculture. In addition to these participants, Dick Guest, Jack Norton and I will be representing IR-4 at the meeting.

WLB:js

Enclosure

cc: R.T. Guest  
J. Norton  
L. Antilla  
P. Cotty  
P. Wakelyn  
E. Minch

New Jersey Agricultural Experiment Station  
Cook College • P.O. Box 231 • New Brunswick, NJ • 08903-0231 • 908/932-9575 • Fax: 908/931-8481

THE STATE UNIVERSITY OF NEW JERSEY

## AGENDA

## EPA PREREGISTRATION MEETING

## MICROBIAL PRODUCT: ASPERGILLUS FLAVUS AF36 ON COTTON IN ARIZONA

Wednesday April 15, 1998 1 PM  
Second Floor Conference Room  
EPA, Crystal Station #1  
2800 Crystal Drive, Arlington, VA

## I. Introductions

Dr. Phillip J. Wakelyn

## II. Aflatoxin in Arizona Cottonseed: A Threat to the Cotton Industry

Mr. Chuck Youngker, Mr. Clyde T. Sharp, Mr. Larry Antilla

## III. Overview of Aflatoxin Biocontrol Studies

Dr. Peter J. Cotty

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uToxicogenicity is reduced without changing the overall quantity of *Aspergillus flavus*

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## IV. Follow-up: Alternatives for Expanded use on Arizona Cotton in 1999.

(The target for 1999 is 20,000 treated acres.)

a a

uActive ingredient production in a publicly managed, grower owned facility.

uData requirements for an amended/expanded EUP.

uRequirements for Section 18 registration.

uRequirements for full registration limited to a public managed program in Arizona.

uOther.

## V. Requirements for Data Waivers for Toxicological and Ecological Effects

## VI. Discussion.

Need a note from

hbee

Daphnia (muff)

Participants from outside the EPA:

Dr. Phillip J. Wakelyn  
Senior Scientist, Environmental Health and Safety  
National Cotton Council

Ms. Carla West  
Government Affairs Representative  
National Cotton Council

Mr. Chuck Youngker  
President Arizona Cotton Growers Association and  
Chairman Arizona Cotton Research and Protection Council

Mr. Clyde T. Sharp  
Chairman Research Committee, Arizona Cotton Growers Association

Mr. Larry Antilla  
Staff Director, Arizona Cotton Research and Protection Council

Dr. Edwin W. Minch  
Environmental Specialist  
Arizona Department of Agriculture

Dr. Peter J. Cotty  
Research Plant Pathologist  
Agricultural Research Service, United States Department of Agriculture

Dr. Jane Robens  
National Program Leader, Food Safety and Health  
Agricultural Research Service, United States Department of Agriculture

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AVIAN  
Honeybee  
Fish  
Daphnia

Differentiate spp. in soil  
Efficacy - up to 100% in regions

Sec 15

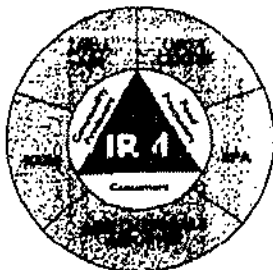
no right reserved

- J. Meredith J. } (i) Write Pat Cimini (owner use)  
D. Pat Cimini } (ii) " Meredith Johnson

A Laurel Celeste

publish ? Feb 11.

B work A = Licence to sell.  
maybe some A. without J. signature



Interregional Research Project No. 4  
Minor Crop Pest Management

April 2, 1998

TO: Phil Hutton  
Shanaz Bacchus

FROM: Bill Biehn *Bill Biehn*

SUBJECT: EPA Preregistration Meeting on Aspergillus flavus  
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WLB:js

Enclosure

cc: R.T. Guest  
J. Norton  
L. Antilla  
P. Cotty  
P. Wakelyn  
E. Minch

New Jersey Agricultural Experiment Station  
Cook College - P.O. Box 231 • New Brunswick, NJ • 08903-0231 • 908/932-9575 • Fax: 908/932-8481

THE STATE UNIVERSITY OF NEW JERSEY

## AGENDA

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hbc

Daphne (Munoff)

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Dr. Peter J. Cotty  
Research Plant Pathologist  
Agricultural Research Service, United States Department of Agriculture

Dr. Jane Robens  
National Program Leader, Food Safety and Health  
Agricultural Research Service, United States Department of Agriculture

DRAFT

8/21/98

Dr. Bill Biehn, Coordinator  
Interregional Research Project No.4  
681 US Highway #1 South  
North New Brunswick, NJ 08902

Dear Dr. Biehn:

Subject: Pre-registration meeting on *Aspergillus flavus* AF36  
for use on Cotton in Arizona

The Agency has reviewed your submission dated May 7, 1998, regarding the minutes of the meeting with certain members of the Biopesticides and Pollution Prevention Division (BPPD). The points in your submission were numbered 1 through 11 to facilitate communication on certain points. The team members have provided the following comments on the meeting:

Item 3 - "The purpose of the EUP.....need to be evaluated."

Your claim in this project is to eliminate the toxigenic strain of *A. flavus*. However, this is accomplished in a gradual manner by displacement competition, which may be better described as a reduction of the aflatoxin contamination in cotton.

Item 7 - "Concern was expressed.....Section 3 registration."

Check with GT (how to present her comment): A blue folder was presented with anecdotal information to support a request to waive data for the avian toxicity studies. Data Waiver requests must be supported by sound scientific information. In your data waiver request, please clarify:

- (a) what preferred habitats for birds are available within the treatment area;
- (b) and the proximity of such habitats to the cotton growing areas;
- (c) what is the exposure of birds likely to be to *A. flavus* AF36 during pesticide application and during the growing season.

Item 8 - "It was also pointed out.....avian toxicology studies."

Clarify whether wheat fields are adjacent to or in close proximity to cotton fields in Arizona.

(Gail, for my own info, what do we need this information for?)

Item 9 - "Since *A. flavus* occurs.....Experimental Use Permit."

(a) The potential pathogenicity of the active ingredient must be addressed. (John: re your statement that "some disclaimer about potential pathogenicity may be needed" what do you mean by your comment and what data/rationale will address your concerns? I need some help here to put your thoughts into words....Thanks) - There will be a lack of path studies to support this use so there may need to be a label warning of potential pathogenicity. appropriate protective equip.

(b) Requests to waive data for environmental fate data must be supported by the submission of appropriate information regarding the microbial ecology of both aflatoxin-producing and non-aflatoxin producing *A. flavus* in Arizona.

Item 11 - "EPA indicated that a description of the manufacturing .....We agreed to provide it." With reference to the folder entitled "Recommendations for setting up a large scale AF-36 wheat production,"

FYI, Shan -

\* Please note: Data are not waived. The requirements for testing may be waived, if appropriate waiver rationale are submitted. Waiver rationale should be accompanied with sound scientific documentation and data.

the team felt that to extrapolate from the scale-up from a 2 cubic feet production process to a 100 cubic feet production process may be problematic and further quality control measures may be required.

It is good that you mention that verification will be requested relative to temperature maintenance within the sterilization procedure for the wheat seeds. However, there is some concern about the presence or proliferation of contaminants during the incubation process. During the scale-up, the time span required for cooling from 37°C to 31°C after inoculum addition may increase. It is feasible that, without adequate Quality Assurance controls, the potential for contamination by extraneous microorganisms can occur. Please describe your Quality Assurance and Quality Control measures to minimize contamination during the manufacturing process. *Since the degree of exposure applies to the hot, sterile seeds*

Also of concern are

- (a) issues about air quality in the manufacturing establishment;
- (b) the transfer of the treated wheat seeds to the drying oven in what may be porous pillow cases; and
- (c) the reference to the possibility that the company may not be aware of the sterile techniques involved in the production process (see memo from Joe Ploski, 4/6/98, to Tom Chirkot Patterson-Kelley Co.).

The steps taken to monitor the air quality in the manufacturing establishment and to implement the use of sterile techniques must be documented and employees must be trained in aseptic techniques??? (HELP, Team! We approve manufacturing methods and product chemistry, OSHA enforces. In the description of the manufacturing method, don't we want information to show that the aseptic techniques will reduce potential contamination of the EP?) *-yes but the seed is applied in a dry, sterile state*

Your communication in writing regarding the above comments will be included in the Agency's assessment of the pending data packages in support of the use of this active ingredient. If you have any questions, do not hesitate to call Shanaz Bacchus on 703-308-8097.

Sincerely,

Phil Hutton  
Chief  
Microbial and Plant Pesticides Branch  
Biopesticides and Pollution Prevention Division

Who signs?

cc: Files

Shanaz

I agree with the comments made by John, particularly regarding the scale-up issue.

Mike W

RE: Comments on "Recommendations for the Setting Up a Large-Scale AF-36 Wheat Production Facility in Phoenix, Arizona, April 1997"

Shan,

I have looked over the *Aspergillus flavus* AF-36 proposal and find that my comments are largely shared by John Kough relative to the scale-up from 2 ft<sup>3</sup> to 100 ft<sup>3</sup>. It is good that they mention that verification will be requested relative to temperature maintenance within the sterilization procedure for the wheat seeds. This could be a pitfall in extrapolating from small scale to large.

My other concern is the presence of contaminants in the incubation process. Given that the incubation period at 30-31 °C is only 20 hours and the moisture content is dropped to 6-8 % (over an undetermined time frame), I don't feel that a major problem exists if the starting inoculum is pure. However, once this process is adjusted to 100 ft<sup>3</sup>, the times for cooling from 37 to 31 °C after inoculum addition may be greater. There doesn't seem to be any QA/QC for extraneous microbes that may be present and problematic. Was this issue addressed during Peter's presentation?

Chris

CAW 8/20/98

Shan,

a/1

re share ~~and~~ John's concerns  
a company that produces equipment for  
sterile processing should be familiar with  
sterile technique. Also, we need to  
make sure there is enough time for  
validation of scale up before final  
approval. - Doug

# ROUTING AND TRANSMITTAL SLIP

Date 8/21/98

TO: (Name, office symbol, building, Agency/Post)	Initials	Date
1. John Kough	JK	8/26
2. Mike Watson	MW	8/27
3. Chris Wormick	CW	8/28
4. Paul Tomu matsu	PT	8/28
5. Doug Curran - Sherman	DC	9/1

Action	File	Note and Return
Approval	For Clearance	Per Conversation
As Requested	For Correction	Prepare Reply
Circulate	For Your Information	See Me
<input checked="" type="checkbox"/> Comment	Investigate	Signature
Coordination	Justify	

## REMARKS

A. flairs (Cotton) Minutes  
of pre Sec 3 meeting.  
Team members: I've assembled  
your comments in a draft letter.  
Please help me to clarify our  
thoughts by commenting on the  
letter before I go final.  
Thanks

DO NOT use this form as a RECORD of approvals, concurrences, disposals, clearances, and similar actions

FROM: (Name, org. symbol, Agency/Post)	Room No.—Bldg.
Sherman	39/CM2/4th
	Phone No.
	308-8097

# ROUTING AND TRANSMITTAL SLIP

Date

9/10/98

TO: (Name, office symbol, room number,  
building, Agency/Post)

Initials

Date

1. Phil Hutton

[Signature]

9/10

2.

3.

4.

5.

Action	File	Note and Return
Approval	For Clearance	Per Conversation
As Requested	For Correction	Prepare Reply
Circulate	For Your Information	See Me
Comment	Investigate	<input checked="" type="checkbox"/> Signature
Coordination	Justify	

REMARKS

A. Hutton AF36  
 Comment on Minutes of  
 the Sec 3 meeting  
 All document from team  
 meeting is incorporated

DO NOT use this form as a RECORD of approvals, concurrences, disposals,  
clearances, and similar actions

FROM: (Name, org. symbol, Agency/Post)

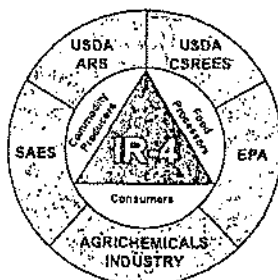
Room No.—Bldg.

Phone No.

[Signature]

916 CPH2

308-8097



**Interregional Research Project No. 4  
Center for Minor Crop Pest Management**

Shanaz Bacchus  
Biopesticide and Pollution Prevention Division  
Document Processing Desk  
Office of Pesticide Programs  
U.S. Environmental Protection Agency  
Second Floor, Crystal Mall 2  
1921 Jefferson Davis Highway  
Arlington, VA 22202-4501  
(703)308-8097

March 25, 2002

Dear Shanaz

RE: *Aspergillus flavus* AF36

As you requested during our conversation on March 20, 2002 I have attached a summary of the toxicology research. Additional toxicology work has been submitted previously, therefore this summary only represents research not yet submitted to EPA. These will be submitted along with the Section 3 registration package.

There have not been any adverse affects attributable to *Aspergillus flavus* AF36 and the primary points of consideration for the continuation and expansion of the EUP and are as follows:

1. The honey bee study determined that *Aspergillus flavus* AF36 is considered non-hazardous.
2. There was no evidence of infectious risk in either avian or mammalian studies
3. There have been no reported adverse effects during the research or EUP phases of product production, development and evaluation.
4. *Aspergillus flavus* AF36 is already found in the soils of the states requested in the EUP.

Technology Centre of New Jersey  
681 U.S. Highway #1 South • North Brunswick, NJ 08902-3390 • 732/932-9575 • Fax: 732/932-8481

5. Aflatoxin is a known toxin and *Aspergillus flavus* AF36 has the ability to reduce this toxin.

Thank you for continued guidance in the EUP process. The EUP for Arizona and Texas will enable additional information to be collected so that EPA can make a better informed decision when the Section 3 registration package is submitted.

Thank you for your time in reviewing this information.

Sincerely,



Michael Braverman, Ph.D  
Biopesticide Coordinator  
IR-4 Project, Rutgers University  
Technology Centre of New Jersey  
681 U.S. Highway 1 South  
North Brunswick, New Jersey 08902-3390  
Tel (732)932-9575 ext 610  
FAX (732)932-8481  
braverman@aesop.rutgers.edu

CC: Phil Hutton, Bob Holm, Peter Cotty

Enclosure- Toxicology Summary

## Preliminary Summary of Toxicology studies on *Aspergillus flavus* AF36

Michael Braverman, IR-4 Project

March 25, 2002

This is a preliminary summary of the results of toxicology studies of *Aspergillus flavus* AF36 in bees, birds and rats performed during 2001 and 2002. We are awaiting the reports with detailed information from the toxicology laboratory (Huntingdon Life Sciences, Ltd. Huntingdon, England). In addition to these studies, through the history of laboratory research, production of *A. flavus*-colonized wheat seed and in field use of this product under the current EUP, there have not been any reported ill effects. This has... included manufacturing personnel, field and laboratory staff, and growers and field... workers. Applications of *Aspergillus flavus* AF36 have been made to commercial fields since 1996 and a total of over 40,000 acres of commercial cotton in Arizona have been treated with *Aspergillus flavus* AF36. Over 400,000 pounds of wheat seed colonized by *Aspergillus flavus* AF36 has been produced at the manufacturing facility in Phoenix. This facility has been developed and built by a partnership between the Agricultural Research Service of the United States Department of Agriculture and the Arizona Cotton Research and Protection Council (ACRPC). The ACRPC is statutory agency of the State of Arizona and is run by a board of cotton producers appointed by the Governor of Arizona in consultation with the Arizona Cotton Growers Association.

Previously submitted information has documented that *Aspergillus flavus* is common on crop and native plants and in soils throughout the areas in which *Aspergillus flavus* AF36 will be applied. Furthermore, it has been documented that *Aspergillus flavus* AF36 is ubiquitous in the areas of Arizona and Texas for which Experimental Use Permits have been requested.

### Material for Toxicology Studies

For all studies *Aspergillus flavus* AF36 was produced in the same manner as when applied to commercial fields for reduction of aflatoxin producing fungi. For the initial mammalian study conidia were produced on sterile wheat seed in sterile bottles just prior to animal dosing. The conidia were washed from the wheat with rigorous shaking in 0.5% Tween 80. In subsequent studies, the avian study and the dose-range study in rat, the conidia were washed from the wheat in sterile physiological saline. For the bee study, colonized wheat seed was applied to a commercial cotton field in the routine manner.

### Honey Bee Study

A study was conducted by The Bee Group of Washington State University. AF-36 colonized wheat seed was applied aerially at 10 lb product/acre to a 40 acre cotton field near Eloy Arizona. This is the rate always used in treatment of commercial fields. A 40-acre control plot was also included. Twelve European Honeybee colonies in the plots were observed for the number of dead bees, number of foraging bees and number of frames of adult bees from 3 to 30 days after application for a total of 24 evaluations.

Researchers used a rating scale in which <100 dead bees/colony /day is considered normal die off (Non-hazardous). Less than 100 bees died (Maximum 86) in all

evaluations in the treated plot. Greater than 100 bees died (122 and 114) in the untreated plots during 2 of the 24 evaluations. There were significantly more dead bees in the treated plots on three of the ratings. There were significantly more dead bees in the untreated plot at one of the ratings. There was no significant difference in the number of bee foragers or frames of bees between the treated and untreated plot. The researchers concluded that AF36 is non-hazardous to bees and can be applied to cotton in bloom with minimal hazard.

#### Avian Inhalation Study

Study was conducted by Huntington Life Sciences, England. Thirty Bobwhite Quail received five mean daily doses of AF36 at  $3.75 \times 10^5$  cfu per bird by intratracheal instillation. Two groups of ten birds were allocated as controls: negative control (five undosed birds and five birds receiving the vehicle) and a heat-killed control.

There were no treatment-related mortalities. Observations over 35 days showed no clinical signs of toxicity and no treatment-related effects evident in either bodyweight change or food consumption. No abnormalities were observed at macroscopic post mortem examination. Treatment with *Aspergillus flavus* AF36 produced no toxicity and no infectivity.

#### Mammalian Studies

The initial pulmonary rat study which resulted in lethality in a significant number of animals treated with either the live *Aspergillus flavus* AF36 in Tween 80 or heat killed *Aspergillus flavus* AF36 in Tween 80. Onset of symptoms was rapid after dosing with all deaths occurring by day four of the study. All rats surviving to day four of the study recovered and all rats sacrificed (as scheduled) on day 8 or day 15 of the study had totally eliminated viable *Aspergillus flavus* AF36 from the lungs, ceacal contents, and faeces. There was no evidence of infectivity. The aetiology of deaths was unclear. However, it is possible that *Aspergillus flavus* AF36 prepared using Tween 80 caused a severe acute inflammatory response. Retrospective literature review and consultation with a toxicologist supported the theory that the responses were a result of a synergism with Tween 80 and/or of Tween 80 breakdown products formed during preparation of the spore suspension.

A second rat study was therefore undertaken. In the second study the conidia were both washed from the wheat and suspended in sterile physiological saline instead of Tween 80. Animals (2 male and 2 female for each treatment level) were dosed at 0,  $10^5$ ,  $10^6$ ,  $10^7$ , and  $10^8$  colony forming units per rat. There were no clinical signs in any of the treatment groups considered to be associated with the test substance. Rats were sacrificed at day 8 without treatment associated mortality. No abnormalities were observed in any of the animals at the macroscopic examination at termination.

Based on these two mammalian studies, we concluded that *Aspergillus flavus* AF36 does not present either a toxicological or an infectious risk to mammals.